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The Development of the Neural Tube of the Chick Embryo. A Study with the Ultraviolet Microscope

by ARTHUR HUGHES[1]

From the Anatomy School, Cambridge

WITH FIVE PLATES

INTRODUCTION

THE main impetus to the study of the nucleic acids and their functional significance within the cell has been mainly derived from the work of Caspersson and his school at Stockholm (Caspersson, 1950). From this source have come the generalizations that relatively high concentrations of ribonucleic acid (RNA) within the cytoplasm are characteristic of rapidly growing tissues, and thus of sites where proteins are being produced. Caspersson has provided evidence that the nucleus is intimately concerned in these synthetic processes. Brachet (1942) came independently to similar conclusions.

These are of importance in a number of related fields in biology, among which is the study of embryonic development. Davidson & Waymouth (1944) compared the nucleoprotein phosphorus of corresponding tissues of the sheep foetus at 9 and 10 weeks with those of the adult animal. They found that only for heart and skeletal muscle were the embryonic concentrations markedly higher on a wet weight basis, but that the values reckoned per unit of dry weight were higher for embryonic than adult tissues in a wider range of organs. Similar results were obtained in comparing 16-day chick embryos with the adult fowl. These authors found, moreover, that these differences between embryonic and adult tissues were due mainly to higher concentrations of polynucleotides in the former and that adult tissues are actually richer in acid-soluble mononucleotides.

Systematic investigation of changes in concentration of nucleic acids throughout the whole or the greater part of embryonic development has so far been confined to the chick embryo (Novikoff & Potter, 1948; Reddy, Lombardo, & Cerecedo, 1952). The work of the first group of authors covers a period of development from the second to the eighteenth day; the RNA concentration is relatively high from the second to the fifth day, at the end of which it suddenly drops, after which it slowly mounts to a further peak at 12–14 days.

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The application of chemical methods to changes within particular organs during embryonic development is clearly a matter of great difficulty, particularly during the early phases when the changes are likely to be of most interest. However, Hermann (1952) has been able to measure the nucleic acids in the somites of the chick embryo during the second and third days of incubation, and Takata (1953) has even been able to estimate by direct analysis the RNA in the ectoderm and mesoderm of the gastrula of *Triturus*.

The alternative methods which can be used to study these changes within tissues and cells are, of course, histochemical. For RNA, staining with basic dyes can be used qualitatively (Brachet, 1940, 1953) or, by use of the microscope in the ultraviolet the absorption of the nucleic acids themselves can be used to reveal their presence. Caspersson's own techniques depend on the use of the ultraviolet microscope. The absorption at particular sites within cells is measured over a range of wavelengths and an absorption curve is plotted. The results are expressed quantitatively, in some instances in terms of absolute amounts of nucleic acids per cell. The technical difficulties, however, are formidable, and in recent years much of the discussion concerning the use of the ultraviolet microscope for microspectrometry has been concerned with the errors involved in such measurements. This subject has recently been admirably reviewed by Davies & Walker (1953). At fixation some material is lost from the tissues (Sylvén, 1951), and the distribution of some of the remainder may be altered both at this stage and during subsequent dehydration. Again, the distribution of light energy in the image plane is affected not only by absorption but by diffraction and scattering; thus 'non-specific' light losses arise, which increase in extent as smaller areas of the object are measured.

The present work is an attempt to follow the general changes in density of RNA at various sites within the spinal cord of the chick embryo during development by ultraviolet photomicrography at a single wavelength. A standard set of conditions has been maintained throughout in order to keep the various losses and errors as far as possible in constant proportions. Apart from non-specific light losses, cytoplasmic absorption at 2,537 Å is very largely due to RNA, though in plant cells there are also present appreciable quantities of ascorbic acid (Chayen, 1952) with an absorption peak in the ultraviolet in this neighbourhood. Ascorbic acid is, however, an unstable substance, and is readily oxidized in warm water with free access to air in the presence of copper. This treatment approximates to the procedure which has here been used in flattening sections, and so it may be assumed that traces of ascorbic acid within tissues in these sections will be decomposed. The main results which have emerged during this work can be deduced from direct comparison of the negatives obtained from material at each stage of development, but it has been thought useful to attempt measurements on the density of various features of these negatives, and to express the results in terms of the apparent optical density of the sections at the particular wavelength used, although for these numerical results only a 'semi-quantitative' status can be claimed. No attempt has been made to use these figures to calculate absolute amounts of absorbing material.

This work is an attempt to continue the studies of Hydén (1943) on the appearance of developing neuroblasts under the ultraviolet microscope. Reference to Hydén's findings will be made at appropriate points in the succeeding pages.

METHODS

Low-power photomicrographs were taken at 2,537 Å of sections of the cord of the chick embryo which had been embedded in ester wax (Steedman, 1947). In the resulting negatives, measurements of optical density were made at particular sites, and expressed in terms of unit thickness of section at this wavelength.

The fixative used was the alcohol-formalin-acetic mixture of Serra (1946) recommended by Brachet (1953) for material which is to be digested with ribonuclease. The quality of the fixation was histologically adequate, although such reagents have considerable effect on the cytoplasmic texture of the differentiated neurone (Hughes, 1954; Koenig & Feldman, 1954). The use of more accurate methods of fixation such as osmic acid or the freeze-drying technique is precluded by the small size of a tissue fragment which can be treated by these means. Moreover, osmium itself has considerable absorption in the ultraviolet (Davies, 1954).

Embryos up to 7 days of incubation were fixed whole, but from later stages the vertebral column with cord and spinal ganglia intact was dissected. Each embryo was carefully compared with the picture and descriptions of each stage given in Lillie (1952). The age corresponding to the stage here identified was taken as the age of the embryo. It was between 12 and 24 hours less than the time of incubation.

After fixation and several washings in 95 per cent. alcohol, the embryo was placed into a mixture of 95 per cent. alcohol and cellosolve (ethylene glycol monoethylether) and then to pure cellosolve. Next it was transferred to a mixture of cellosolve and ester wax at 45° C., and then to pure ester wax in which the embryo was finally embedded. For a 3-day chick the whole process need occupy no more than 2–3 hours.

For section-cutting the ultra-microtome version of the Cambridge rocker was employed, set for the maximum thickness of cut, which was 2·7 μ. The razor-blade adaptor supplied with the microtome was used, modified to allow the sections to be floated on a water meniscus. The blade and its holder were cooled with ice during use. The block was trimmed to the desired level within the embryo, and sections were cut until they came regularly off the blade and were of even appearance. A few of these were then mounted on quartz slides and dried. Finally, they were mounted in medicinal paraffin under a quartz coverslip. The only variation in this procedure was made when it was required to submit the section to the action of ribonuclease. Here the sections were first mounted in distilled water, when some loss of sharpness in photography resulted from the use of a fluid of refractive index relatively low for this purpose. The sections were then dried and immersed in ribonuclease solution for various periods, after which the photographic procedure was repeated.

The ultraviolet equipment was supplied by Messrs. Cooke, Troughton, & Simms Ltd. The microscope is a standard vertical instrument to which a large drum has been fitted to the fine adjustment control, and is calibrated in microns. The sub-stage mirror was aluminized.

The source of radiation at 2,537 Å was a mercury resonance lamp mounted in a housing which also contained a cadmium arc used for other wavelengths in the ultraviolet, together with the necessary quartz prisms for isolating these emission lines.

The preparation was first focused in a side-viewing tube of the microscope with a × 10 phase-contrast objective, and the desired field was selected. A suitable annulus was placed below the quartz condenser, and an ordinary lamp interposed between the microscope and the ultraviolet illuminator.

The objective was then changed to the × 10 quartz monochromat, the phase ring swung out of the field, and the eyepiece replaced by a fluorescent one. The sub-stage condenser could now be focused and the field iris brought into the plane of focus of the object. The fine adjustment of the microscope was then moved through a calibrated distance, the side-viewing tube of the microscope withdrawn, and a series of exposures made at a magnification of × 40 on Ilford 'Safety Positive' 35 mm. film, between each of which the fine adjustment was moved on by 5 μ. Within a series of six exposures made under these conditions the point of exact focus can usually be found with out-of-focus images to either side. The field of focus is curved, and detail towards the margin of the negative is usually sharper in an adjacent exposure. From time to time a × 81 glycerin-immersion monochromat was exchanged for the × 10 objective, and thus high-power photographs were taken with the maximum possible resolution under these conditions (Plate 5).

On the same length of film an exposure was also made of a rotating step-wedge. This consists of an opaque disk, segments of the circumference of which have been removed to reduce the amount of ultraviolet light successively to $\frac{1}{2}$, $\frac{1}{4}$, and $\frac{1}{8}$th of its full intensity. This was swung into place in front of the field lens, the section was moved out of the field, and the image of the rotating disk focused in the object plane was then photographed though the quartz optical system of the microscope.

The film was then developed under standard conditions of time and temperature with continuous agitation. Ilford developer ID19 was used for this purpose. When fixed, washed, and dried, the film was ready for measurements of optical density, for which an 'Ilford SE1' photometer was mounted on a microscope stand and used with a low-power objective.

This instrument consists of an eyepiece, in the centre of the field of which is a spot illuminated by a lamp in a side-tube, before which a system of neutral wedge filters can be moved by an external graduated control. The photometer eyepiece was mounted in the axis of the objective, so that the ultraviolet negative could be placed on the stage of the microscope, illuminated with nearly parallel light by the sub-stage mirror, and viewed through the photometer eyepiece at a magnification of about ten diameters. It was thus possible to place near the illuminated spot the image of a nucleolus, a mitotic figure, or of a point in the cytoplasm of a neuroblast, and match the two together by rotating the neutral filter control, which is graduated in optical densities over a wide range. A measure of the density of the particular image detail could be obtained by subtracting the value measured at the spot from the background density at a nearby point where the section was 'empty'. In a photograph of the spinal cord the central canal provided such a point when measurements were being made within the ependymal layer. Care was always taken throughout this work to confine readings within a cell to points of maximum density. Measurements were made in groups of three, and the mean value was calculated. Also in the negative the density of each step of the rotating wedge was measured in the same way, and the values plotted. Optical densities are expressed on a logarithmic scale; the abscissal values were also plotted in the same way with an interval of 0·3 between steps, each of which represents a halving of the incident light intensity. The slope of this curve is known as 'gamma': it is a measure of the contrast of the photographs under the particular conditions of development, which are assumed to be constant for all exposures in each length of film. Such characteristic curves are straight lines except at the extremes, which bend sigmoidally. Exposures taken of sections under the microscope were chosen to lie within the straight part of the gamma curve. The contrast of the emulsion is much less in the ultraviolet than in the visual range of the spectrum; under the conditions of the present work gamma values varied usually between 0·7 and 0·8.

By dividing the densities measured in the negatives by the appropriate value of gamma, the density of the corresponding point in the section to ultraviolet light at 2,537 Å is obtained. The results were expressed finally as density per micron thickness of section. At various stages of development such measurements were made for the cytoplasm of the ependymal cells and of the neuroblasts; in dorsal root ganglia and in the ventral horn; as well as for the ependymal mitotic figures, and the nucleoli of the dorsal root ganglionic neuroblasts.

The results bear no relationship to the total content of nucleic acid per cell, about which no information is yet available. The present data are some measure of the amount of absorbing material per unit volume of embryo, though the unit taken is a very minute fraction of the whole. However, as will be seen below, they bear some relation to the density of RNA per unit of wet embryo weight, which approximates to concentration per unit volume.

OBSERVATIONS

(*a*) *General changes*

In Text-figs. 1 and 2, values for optical density per micron thickness of section at 2,537 Å are plotted against time of incubation for points of maximum density within the cytoplasm of neuroblasts of dorsal root ganglia (1*b*) and ventral horn cells (1*c*), of ependymal cells (2*b*), and also for the density of mitotic figures of the germinal layer (2*a*). Sections of the cord at brachial levels have been used throughout for measurements on ventral horn cells and on the neuroblasts of the dorsal root ganglia. In the latter the more differentiated neuroblasts were selected. This is of importance towards the end of the first week of incubation, when a ventro-lateral group of cells is markedly more advanced in development than the remainder (Hamburger & Levi-Montalcini, 1949). In Text-fig. 1*b*, the first set of values, just before 2 days of incubation, refer to cells of the undisturbed neural crest. Measurements are restricted to the first two weeks of development; afterwards, as Nissl substance accumulates in the larger neurones they exhibit very large variations in density of absorbing material, the distribution of which is affected by fixation (Hughes, 1954). In Text-fig. 1 are also included the data given by Novikoff & Potter (1948) for the concentration of RNA in the whole embryo in mg. RNA/gm. of wet weight.[1] In Text-fig. 1 the data for RNA concentration in the whole embryo (*a*) and for the cytoplasmic density of sensory (*b*) and motor neuroblasts (*c*) have one striking feature in common, namely, a sudden fall in values between the fifth and sixth days of development. In the negatives on which the measurements in Text-fig. 1*b* and 1*c* are based, the difference in contrast between those of 5 and 6½ days of incubation is very marked indeed, though the distinction has necessarily become somewhat toned down in plates prepared from these negatives (Plates 1–4). After the sixth day in all the data of Text-fig. 1 there is a slow rise once more.

The similarity between these absorption measurements and the data for the RNA concentration in the whole embryo suggests that for the first week of development Novikoff & Potter's curve relates mainly to changes within the nervous system, which must presumably contain a large proportion of the RNA of the whole embryo. This conclusion is supported by inspection under the ultraviolet microscope of sections of chick embryos during the first few days of incubation when the nervous system is much the most prominent feature. Davidson (1950, p. 68) remarks that neither the heart nor the liver seems responsible for the changes in RNA concentration during the development of the chick; absorption measurements in sections of the liver do not suggest any general trend in variation in density from 5 days onwards.

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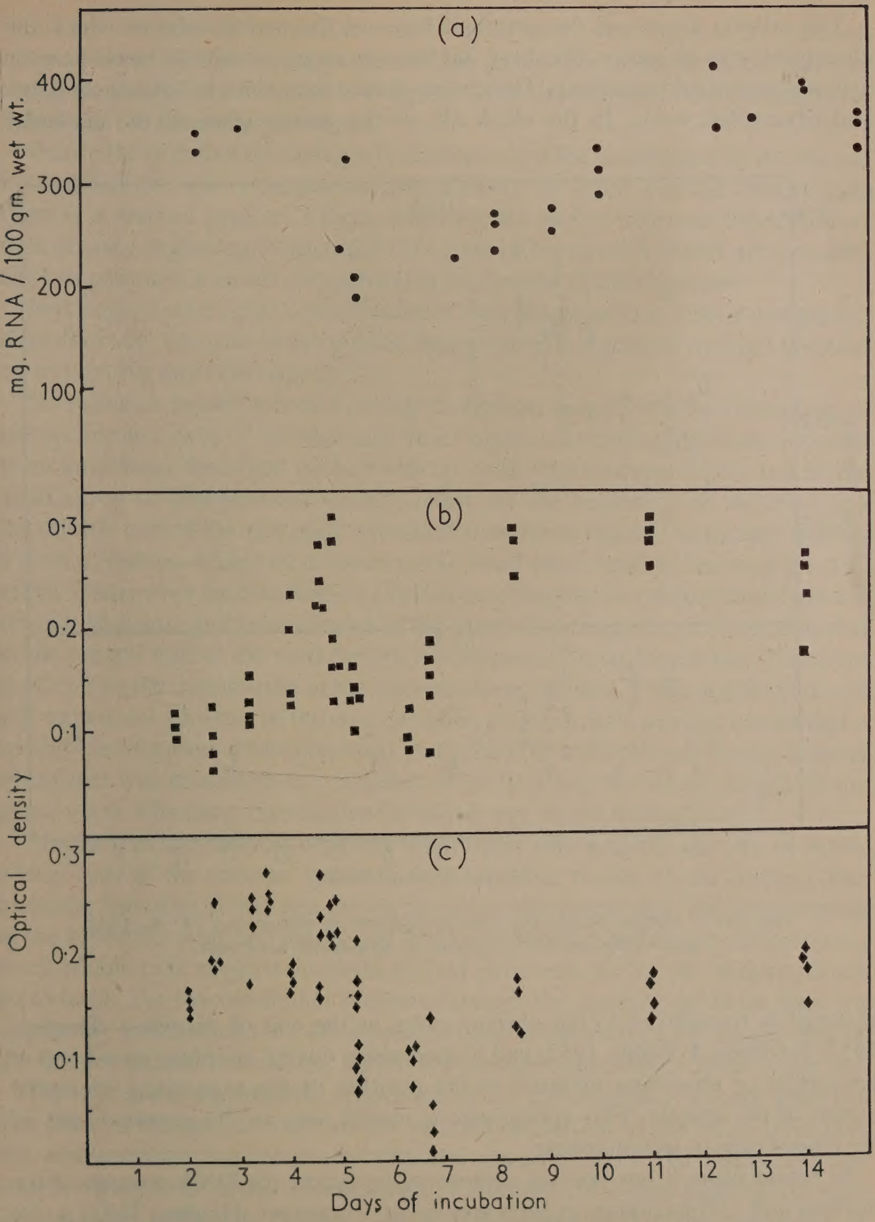
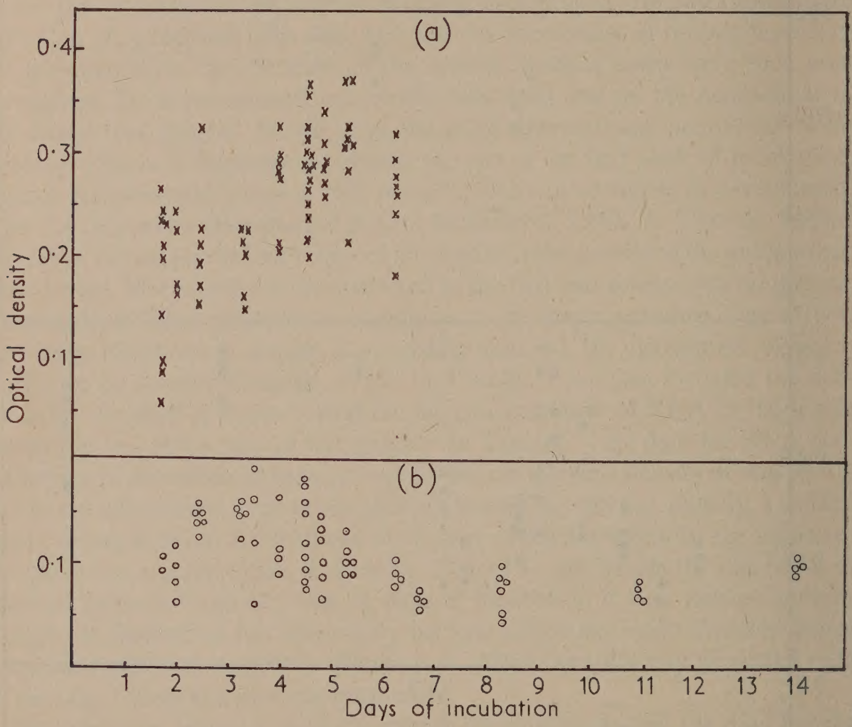
[1] Dr. Novikoff has been kind enough to inform me that in Fig. 3 of their paper the data on the RNA content of the chick embryo are plotted on a wet weight basis, and not on dry weights as stated. Reddy *et al.* (1952) have used a dry weight basis for their RNA values from the seventh day of incubation onwards, in which the concentration remains unchanged from this stage until 13 days, after which it steadily decreases.



TEXT-FIG. 1. (*a*) Novikoff & Potter's data for RNA concentration in the whole embryo in mg. RNA/100 gm. embryo wet weight. (*b*) and (*c*) Optical density per micron thickness of section at 2,537 Å of cytoplasm in (*b*) neurones of dorsal root ganglia. (*c*) Ventral horn cells. All plotted against days of incubation.

The mitotic figures of the germinal layer of the neural tube of which the absorption was measured (Text-fig. 2*a*) were at stages of mitosis between early metaphase and mid-anaphase. The chromosomes then contain both desoxyribo- and ribonucleic acids. In the chick all, or the greater part, of the nucleolar material is transferred to the chromosomes at the end of prophase (Hughes, 1952; Jacobson & Webb, 1952) and is shed again during anaphase as a band of basophilic or absorbing material in the position of the expanding interzonal region of the spindle. This appearance is readily seen in the germinal cells of the neural tube in late anaphase.



TEXT-FIG. 2. Optical density per micron thickness of section at 2,537 Å of (*a*) mitotic figures, (*b*) cytoplasm of ependymal horn cells.

In recent years it has become customary to regard the DNA content of the nucleus and chromosomes in each species as a constant (Hughes, 1952, p. 35) though this has yet to be proved true for the whole period of embryonic development in any organism. It is possible that the indication of a peak in absorption of the mitotic figures of Text-fig. 2*a* at 4–5 days of incubation is due to their RNA content in conformity with the data on cytoplasmic absorption in Text-fig. 1;

this view is supported by measurements which have been made on the nucleoli of neuroblasts of dorsal root ganglia which follow the same trend. Further evidence on the RNA of the mitotic figures from experiments with ribonuclease follows in the succeeding section.

Before the fifth day the changes in absorption of the neuroblasts of the ventral horn and of the spinal ganglia are not precisely the same. For the sensory cells there is a marked peak at 5 days, while for the motor neurones absorption is then already beginning to decrease. The data of Novikoff & Potter are not sufficient to permit of a closer comparison at this period of development.

In the ependymal cells the absorption of the cytoplasm is at a maximum at 4–5 days (Text-fig. 2*b*), and in the mitotic figures there is a general trend of increase up to the fifth day (Text-fig. 2*a*).

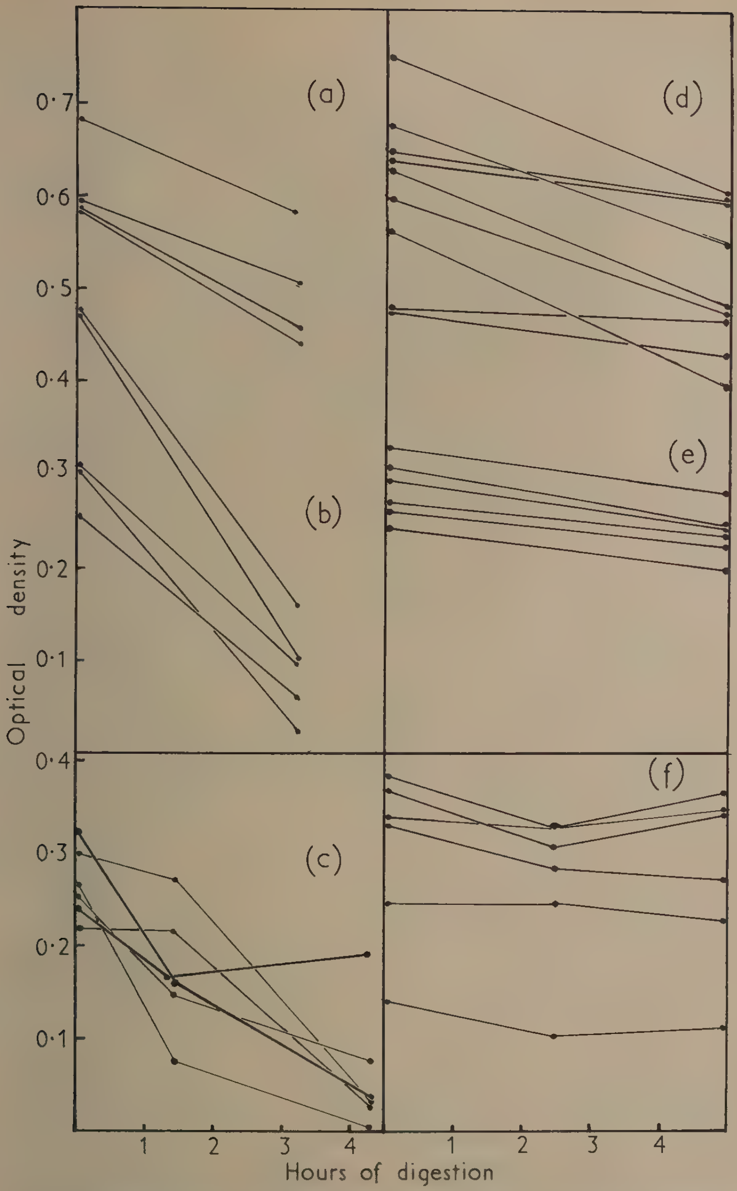
No evidence could be found of any distinction in cytoplasmic absorption of the ependymal cells of the alar and basal plates, although differences between them have been described in their mitotic rates (Hamburger, 1948), and in the distribution of phosphatases (Moog, 1943). At the beginning of the third day the mitotic rate of the alar plate overtakes that of the basal. The former reaches a peak at 6 days, while cell division in the basal plate steadily becomes less frequent. Changes in the distribution of alkaline phosphatase proceed mainly in the reverse direction, and from the end of the fourth day this enzyme is concentrated in the ventral half of the cord though it is absent in the motor horns. There are gradients in the distribution of the enzyme along the cord. I was unable to detect any such axial gradient in the concentration of RNA; at 4 days of incubation a special investigation of this point was made, and the absorption of the ependymal cytoplasm was measured at a number of levels along the whole length of the spinal cord. The same distribution of values was found throughout.

The lack of correlation between the mitotic rate and the density of RNA within cells of the nervous system is true for other tissues of the embryo. For instance, Saunders (1949) has shown that from the fourth day, the mesenchyme of the limb-bud is all derived by cell division within an apical cap of ectoderm, from which cells migrate inwards to add progressively to the skeletogenous mesoderm. Yet the ultraviolet sections through the apex of the limb show an apparently uniform density of cytoplasm both in the cells of the apical cap, in the thinner adjacent ectoderm, and in the mesenchyme within (Plate 3, fig. M).

The increase in cytoplasmic absorption of the differentiated neurone after the first week of incubation is associated with the deposition of the Nissl substance; the characteristic basophilic cytoplasmic constituent of the adult neurone which, owing to its ultraviolet absorption (Hydén, 1943) and digestibility with ribonuclease (Gersh & Bodian, 1943), is regarded as largely composed of RNA. It differs, however, from the RNA of earlier stages in giving a violet or β-metachromatic reaction to basic dyes. This is clearly obvious in the chick at about 9 days of incubation in sections stained in thionin buffered at pH 3·5 (see p. 319 below). There are three possible interpretations of the metachromasia of the Nissl substance. It may be due to polymerization of the dye as a result of staining a highly polymerized nucleic acid (Michaelis & Granick, 1945; Michaelis, 1947). The other possibilities are that either the phosphate groups of the RNA are responsible, or a highly polymerized carbohydrate associated with the Nissl substance (Pearse, 1953, p. 149). Even a small degree of metachromasia may be due to different causes in different cells; Wislocki, Bunting, & Dempsey (1947) have shown that ribonuclease abolishes this reaction to basic dyes in neurones but not in megakaryocytes.

Experiments with ribonuclease. For this work the method used is that recently described by Brachet (1953), namely, incubation of sections at 37° C. in ribonuclease dissolved in distilled water adjusted to pH 6. The ribonuclease was omitted in the control experiments, but all other features were maintained unvaried. The crystalline ribonuclease was supplied by the Worthington Biochemical Co. of New Jersey, and prepared by the standard methods of Kunitz (1940) and McDonald (1948). Owing, however, to the fact that ester wax is permeable to water, it was found unnecessary to remove it from the mounted sections in these experiments, but the incubation times were accordingly increased for 3–5 hours.

Sections were mounted in water on quartz slides and photographed at 2,537 Å They were then dried and incubated at 37° C. at pH 6·0 either with ribonuclease at concentrations which varied from 0·1 to 1·3 mg./ml., or in control experiments with water alone. In parallel experiments on the same material with different concentrations of ribonuclease, no difference in effect could be observed within this range of concentrations. The sections were then washed, dried, and again mounted in water and photographed, after which in some cases digestion was repeated, and followed again by photography. The resulting negatives were then compared, and the density of the same site in each of them was measured for a number of points in mitotic figures and cytoplasm. The results of two sets of such experiments are shown in Text-fig. 3. They relate to (*a*) mitotic figures, and (*b*) cytoplasm of ependymal cells in the 4-day embryo, and (*c*) the neurones of a dorsal root ganglion in a newly hatched chick. The results of digestion in distilled water at pH 6 in observations which serve as controls to each of these three sets of experiments (*a*–*c*) are shown in (*d*–*e*) respectively. For mitotic figures, some of the absorbing material is apparently as soluble in water as in ribonuclease, whereas in cytoplasm there is considerable difference between the effect of the two media. Other experiments have shown that incubation continued after 3–5 hours results in little or no further decrease in the absorption of mitotic figures. There is no evidence that all the material extracted from chromosomes consists only of ribonucleotides, though the interpretation of these experiments may well be that the RNA of the chromosomes, so rapidly deposited thereon at the end of prophase and so readily removed in early anaphase, is less highly polymerized than is the RNA elsewhere in the cell. Other experiments have shown the nucleoli of intermitotic cells are as resistant to the action of water alone as is cytoplasmic RNA.



TEXT-FIG. 3. Optical density per micron thickness of section at 2,537 Å before and during treatment with ribonuclease at 37° C. of (*a*) ependymal mitotic figures, and (*b*) ependymal cytoplasm at 3½ days (0·1 mg. ribonucleases/ml.). (*c*) Nissl substance in dorsal root ganglionic neurone at 31 days (1·3 mg. ribonuclease/ml.). (*d*), (*e*), and (*f*) refer to sections treated with water to serve as controls to (*a*), (*b*), and (*c*) respectively. All plotted against hours of digestion.

(*b*) *The differentiation of the neuroblast*

In this section will be described the appearance in ultraviolet photographs of differentiating neuroblasts, both in the cord and ganglia. Some comparison will be made with the aspect of the same cells in silvered preparations both as described and figured by Ramón y Cajal (1909), Tello (1922), Windle & Orr (1934), and Barron (1946); and also as seen in series of sections prepared during the course of the present work. For these the silver-on-the-slide methods of Holmes (1947) and Bodian (1936) were used. Holmes's method is excellent for the earliest stage of differentiation, although the protargol technique is preferable at later stages.

(i) *Within the cord.* The earliest stage in the cord at which I have seen nerve fibres impregnated with silver is 50 hours (Plate 1, fig. B). The embryo of 46–48 hours of which Tello (1922) describes the cord at cervical levels corresponds to stages 15–16 in the Hamilton-Lillie series, and to a standard time of incubation of 50–56 hours. Longitudinal fibres, commissural, and motor neuroblasts are all present in the cord at this stage.

In Plate 1, fig. B the differentiating neuroblasts at the lateral surface of the cord are just emerging from their primitive bipolar condition, for in some a medial process can also be seen which is very much less impregnated with silver than is the main fibre. In the ultraviolet (Plate 1, fig. A) these neuroblasts which have begun to differentiate can be recognized not only by their position, but also by the fact that absorbing material in the cytoplasm has begun to accumulate round the nucleus. At 58 hours (Plate 1, fig. C) this appearance is maintained, and it can be seen that in some differentiating neuroblasts the nucleus is enlarged and the nuclear sap is lighter than in cells elsewhere in the neural epithelium. By 3½ days (Plate 1, fig. E) the most advanced group of neuroblasts, which represent the future somatic motor neurones of the ventral horn, have become markedly distinct from the surrounding cells. Strongly absorbing material is accumulating round the nuclear membrane; in some cells this is heaped up on one side to give the appearance of a dense cap, such as Hydén (1943) has already described for differentiating neurones in the cord of a rat foetus at a length of 10–13 mm. In the chick, from 3½ days onwards, different groups of neuroblasts at various sites assume this appearance some time after they have begun to differentiate. At a later stage the perinuclear cap becomes less concentrated as the absorbing material seems to spread outwards along the nerve fibres. For the first group of somatic motor neuroblasts this has already occurred at 4½ days (Plate 1, fig. F); other neuroblasts are being added to this group; dorsal to them is a prominent group of commissural neuroblasts, each with the dense perinuclear cap. At this stage general absorption is rising towards the maximum both in the fibres of the white matter, and in the cell bodies. The peak is reached some 6 hours later in the fifth day (Plate 2, figs. G and H) when every fibre in the white matter can then be distinguished at 2,537 Å. The perinuclear cap of the younger neuroblasts then approaches in density that of the mitotic figures of the germinal layer. At this stage also, the rate of differentiation of neuroblasts is at a maximum, and the process has spread deep into the mantle layer of the cord. Some of these secondary neuroblasts (Barron, 1946) are joining the somatic motor column; their axons may emerge from the cord through either ventral or dorsal roots (Tello, 1922; Windle & Orr, 1934). The majority of these newer neuroblasts are, however, commissural. In many of these the density characteristic of the perinuclear cap extends for some distance along their fibres as they sweep downwards and inwards towards the ventral commissure. These newly differentiated neuroblasts are of various sizes; those of the alar plate are generally much smaller than those which are basal in position.

At this time high-power photomicrographs at 2,537 Å taken with a glycerin-immersion lens reveal that the nuclei of the ventral horn cells are markedly granular in appearance (Plate 5, fig. R) and resemble nuclei in prophase, although they are, of course, well past their early mitotic period within the ependymal zone. The distinctness of the chromosome threads at this period suggests that much of the DNA of the nucleus is concentrated upon them, though the significance of this fact is as yet obscure.

When the sixth day is reached, the peak of activity within the cord which we have described is past. Absorption in the perikarya of the neurones falls to a minimum and gradually declines within the white matter. Towards the end of the seventh day (Plate 3, fig. J) the perikarya of the ventral horn are less dense than are the erythrocytes within the capillaries of the cord. New groups of neuroblasts continue, however, to differentiate; they are now most prominent in the developing dorsal horn, but at 6 days (Plate 2, fig. I) a group within the basal plate close to the ependymal layer can be recognized at thoracic levels. This corresponds with a visceral motor centre which has been described by Levi-Montalcini (1950).

From 7 days onwards the motor neurones of the ventral horn begin to enlarge and to accumulate new RNA which soon takes the form of Nissl substance. At the stage of hatching (Plate 4, fig. Q) the largest motor neurones have increased more than five times in diameter from their original size, and are now much more densely absorbing than at any previous stage. Their content of RNA may well have increased a thousandfold.

(ii) *In the dorsal root ganglia.* At cervical and thoracic levels of the cord, the cells of the neural crest which are destined to form the dorsal root ganglia migrate from the crest to their final position during the third day. At the end of this period, some of these cells have become bipolar neuroblasts, and at brachial levels their efferent fibres have reached the surface of the cord but have not yet entered it (Tello, 1922, Fig. 33). They are preceded by motor fibres which emerge from the cord in a dorso-lateral position (Tello, 1922, Fig. 32). While still within the crest the density of these cells in the ultraviolet is only slightly greater than in those elsewhere in the neural tube (Plate 1, fig. A). By the time that they have migrated to their final position their absorption in the ultraviolet has increased well beyond that of the surrounding mesenchyme cells (Plate 1, fig. D). In these sensory neuroblasts, this increase is roughly contemporaneous with fibrillation, and the first signs of their affinity for silver. During the fourth and fifth days the cytoplasm of the ganglionic neuroblasts increases markedly in volume without any increase in the size of the nucleus (Plate 1, fig. F). RNA is not concentrated in a dense perinuclear cap, but is evenly distributed throughout the cytoplasm, and during the fifth day is sufficiently dispersed along the fibres to allow them to be distinguished in an ultraviolet photograph.

By this time the perikarya have begun to differentiate in the way described by Levi-Montalcini & Levi (1943) and by Hamburger & Levi-Montalcini (1949); and the nuclei of a few neuroblasts have begun to enlarge (Plate 1, fig. F). By 5 days of incubation larger neuroblasts are prominent in the ventro-lateral region of the ganglion. Plate 2, fig. H shows the upper part of a cervical ganglion at this stage. Cells of various sizes are present, some of the smaller of which show a very high degree of ultraviolet absorption. Pycnotic cells are common (section (*c*) below).

The section from the thoracic region of the cord during the seventh day illustrated in Plate 2, fig. I includes, on the right-hand side, part of the ventral region of a spinal ganglion. The larger neuroblasts are beginning to show some of the characteristics of ganglionic neurones at later stages of incubation as the density of absorbing material begins once more to increase after the minimum at the sixth day. The nucleus becomes eccentrically placed, while a large adjacent area contains very little RNA. Dense absorption is confined to circumscribed zones at the periphery which on one side may surround part of the nuclear membrane. These features become more prominent in later stages of development (9 days, Plate 4, fig. N; 21 days, Plate 4, fig. P; Plate 5, fig. S). The absorption of the developing Nissl substance during the second week of incubation becomes equal to that of the denser neuroblasts during the earlier peak at the fifth day.

(*c*) *Cell degeneration within the developing nervous system*

In 1906 Collin described the appearance of degenerating cells in the developing ventral horn of the chick cord between 4 and 6 days of incubation. Hamburger & Levi-Montalcini (1949) confirmed and extended these observations. Degenerations then appear also among neuroblasts of the dorsal root ganglia, and in both, cord and ganglia are most frequent at non-limb levels, where they serve to ‘thin out’ the neurones and so effect a reduction in calibre below that of brachial and sacral levels. Among the densely staining pycnotic cells and nuclei are found macrophages, which remove cell debris by phagocytosis. It is possibly significant that these events follow the first vascularization of the neural tube (Feeney & Waterson, 1946).

My own observations confirm these findings with respect to the distribution and time of appearance with the cord and dorsal root ganglia of pycnotic cells.

These show a very heavy concentration of absorbing material in the ultraviolet. They are first found when the density of RNA within the embryo generally and in the cord is approaching its first maximum (Plate 1, fig. F; Plate 2, fig. H) and persist into the period of the subsequent minimum (Plate 2, fig. I). Whether this feature of neural development plays any special part in the turnover of the nucleic acids must remain to be investigated.

(*d*) *The development of Nissl substance in stained preparations*

Several authors have studied the basophilic material of the larger neurones of the chick and spinal ganglia in the later stages of development, among whom may be mentioned Collin (1906) and Marcora (1911). In ganglionic neurones Collin found that fine granular material is present at 5 days of incubation, and gradually increases in amount to the eleventh day. At 14 days this basophilic substance has been aggregated into 'Nissl bodies'. According to Marcora this material first takes this form at about the tenth day.

These earlier authors, who used simple dilute solutions of basic dyes, did not notice the metachromatic reaction of the Nissl material which is so marked a feature when solutions of these reagents buffered at an appropriate pH are used (Windle, Rhines, & Rankin, 1943). This reaction provides a better criterion for the first appearance of Nissl substance than does the manner of its aggregation, which is subject to the influence of the fixative agent (Einarson, 1935; Hughes, 1954; Koenig & Feldman, 1954).

In the larger neurones of the cord and of the dorsal root ganglia of the chick this metachromatic reaction is obvious at 9 days of incubation. In the ganglionic neurones there is a striking contrast between the central area next to the nucleus which stains a light clear blue colour, and the adjacent dense purple masses of Nissl material.

Comparison of the appearance of neurones of later stages of development in the ultraviolet with their appearance after staining with basic dyes does not reveal any essential difference in the distribution of the Nissl material. However, as Einarson (1935) has shown, the basophilic material does not consist of 'chromatin' alone, but also of basic proteins, which within the living cell are presumably combined as nucleoproteins.

(*e*) *Microglia*

A new feature within the white matter of the cord is to be observed from the ninth day. Before this period the only cells which can here be seen are a few radially elongated ependymal elements (Plate 2, fig. I). At 9 days, however, small densely absorbing cells invade the white matter from the surrounding meningeal tissue (Plate 3, fig. L), which at this time has undergone some differentiation from the loose mesenchymatic condition of previous stages. The meningeal cells are now tangentially orientated, and among them are extremely dense elements which appear to correspond with those which have already entered the cord. At 11 days (Plate 4, fig. O) these cells still show the same distribution, but by the hatching stage they have spread everywhere into the grey matter (Plate 4, fig. Q), where they present a less dense appearance with somewhat larger nuclei than at earlier stages.

These cells, from their manner of entering into the cord, may be identified as microglial elements (Penfield, 1928; Del Dio Hortega, 1921). Characteristic of them is a relatively dense chromatin within the nucleus, and marked affinity for toluidine blue.

These observations are not inconsistent with the view that in the chick such cells may be derived from the neural crest (Harvey & Barr, 1926), for similar small dense cells can be seen in the neighbourhood of the dorsal root ganglia at stages before their appearance within the meninges and cord.

DISCUSSION

In preceding pages sufficient attention has already been drawn to the similarity of the changes in the concentration of RNA in the whole embryo with those in the density within the cells of the nervous system. It remains to be pointed out that the early period of high RNA values up to the fifth day of development is contemporary with the stages when the peripheral nervous system is first sketched out. The cranial nerves are mainly formed during the fourth day (Lillie, 1952); the brachial nerves can be traced into the wing bud on the fifth day (Barron, 1946), and at the same stage the permanent sympathetic trunk is developed (Tello, 1925).

The successive changes in density of RNA within the cells of the nervous system from a high level to a low, followed by a slow increase once more, seems broadly to hold for individual neurones, as well as for the cord as a whole. The timing of these events is not the same for all neurones however; the various groups develop at different periods, and their initial differentiation is closely followed by an early high level of RNA density.

The obvious question is how far these facts apply to the development of the nervous system in vertebrates other than the chick. Hydén's original work in this field (1943) suggests that the individual neurones of the rat cord go through a similar cycle, but at present insufficient is known about changes in RNA concentration within other relatively early embryos to enable us to form any idea whether the shape of this curve for the chick is related mainly to the conditions of avian development. Nucleic acids are present in the yolk of the hen's egg, and thus a ready supply of this necessary formative material is available for the early embryo.

The only relevant evidence elsewhere comes from the studies of Flexner and his colleagues on the cerebral cortex of the foetal guinea-pig (reviewed in Flexner, 1950). In recent years the work of this school has revealed much information on the enzymatic and histochemical changes associated with the development of this tissue.

Among the various aspects of the differentiation of the cortex which have been described by these workers, are variations in the RNA content of the individual cortical cell (Flexner & Flexner, 1951). These values have been calculated by combining data on nucleic acid phosphorus and on the amount of perikaryon per unit weight of the cortex (Peters & Flexner, 1950), making the usual assumption that the DNA content of the nuclei is a constant. The resulting curve for RNA phosphorus per unit weight of perikaryon shows a sharp decrease from the thirty-eighth day to a minimum between 55 and 60 days, followed by a further period of increasing values. During this cycle there comes a critical period of chemical differentiation from the forty-first to the forty-fifth days, when, among other synthetic activities, the Nissl substance is very rapidly formed (Peters & Flexner, 1950). It is therefore likely that if values for the density of RNA within the developing cortical neurones were plotted for the densest areas within the cells, there would result a curve similar to those which have been obtained for the chick cord, with a sharp fall and a subsequent rise during this critical period from 41–45 days. In other types of cell rapid synthesis of RNA coincides with a period of function differentiation; thus in the chick liver (Plate 3, fig. K) a perinuclear ring is seen most clearly in ultraviolet photographs at 6–6½ days, a time within 24 hours of the stage when, according to Dalton (1937) many of the hepatic cells have become capable of active mature function.

So far the only enzyme for which the chick cord has been assayed is acetylcholinesterase (Wenger, 1951). Here there is a general correspondence with the data from the foetal cortex of the guinea-pig (Kavaler & Kimel, 1952). In the latter, activity begins rapidly to increase at the thirty-fifth day, some 2 days before RNA phosphorus commences to decrease; in the chick cord acetylcholinesterase increases from the fifth day, which is just before the general drop in RNA concentration.

One possible point of difference between comparable data for the guinea-pig cortex and the chick embryo, however, may be in regard to their protein contents. In the latter the sixth day represents a minimum not only in RNA concentration, but also in percentage of protein (Needham, 1931, vol. II; Novikoff & Potter, 1948), although it is not known whether the nervous system follows the whole embryo in this respect. In the guinea-pig cortex the protein concentration remains uniform over most of the period of gestation, but shows a slight rise just before term (Flexner & Flexner, 1950).

It must remain for further work to determine how far the development of nervous tissue in different species is comparable with respect to these various features of chemical differentiation.

SUMMARY

1. The development of the spinal cord and dorsal root ganglia of the chick has been studied by ultraviolet photomicrography of sections at 2,537 Å. The results have been compared with material treated by standard neurohistological methods.

2. In the ultraviolet negatives cytoplasmic density for unit thickness of section has been measured for ependymal and ventral horn cells, and also in the neuroblasts of the dorsal root ganglia. The density of the mitotic figures in the ependymal layer has also been measured.

3. The changes in cytoplasmic density in developing neurones closely parallel those in the RNA concentration of the whole embryo.

4. In the developing neurone a high density of RNA is seen within the cytoplasm for some days after the cell has first differentiated. This then falls and later rises slowly once again as Nissl substance is laid down.

5. Experiments on the effect of ribonuclease on ependymal mitotic figures and the cytoplasm of neuroblasts are described.

ACKNOWLEDGEMENTS

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EXPLANATION OF PLATES

With the exception of Plate 1, fig. B, all the photomicrographs here reproduced are of unstained sections in the ultraviolet at 2,537 Å. A standard magnification of ×250 is used for Plates 1–4; for Figures R–S of Plate 5 a glycerin-immersion lens was used of N.A. 1·25 and the final magnification here is ×2,000.

A key is provided for Plates 1 and 2, in which recently differentiated neuroblasts are outlined, and pycnotic nuclei are shown in solid black. The abbreviations used are as follows:

c.c.	central canal	n.	notochord
d.m.t.	dermatome	n.c.	neural crest
d.r.g.	dorsal root ganglion	p.n.	primary neuroblasts
m.y.t.	myotome		

PLATE 1

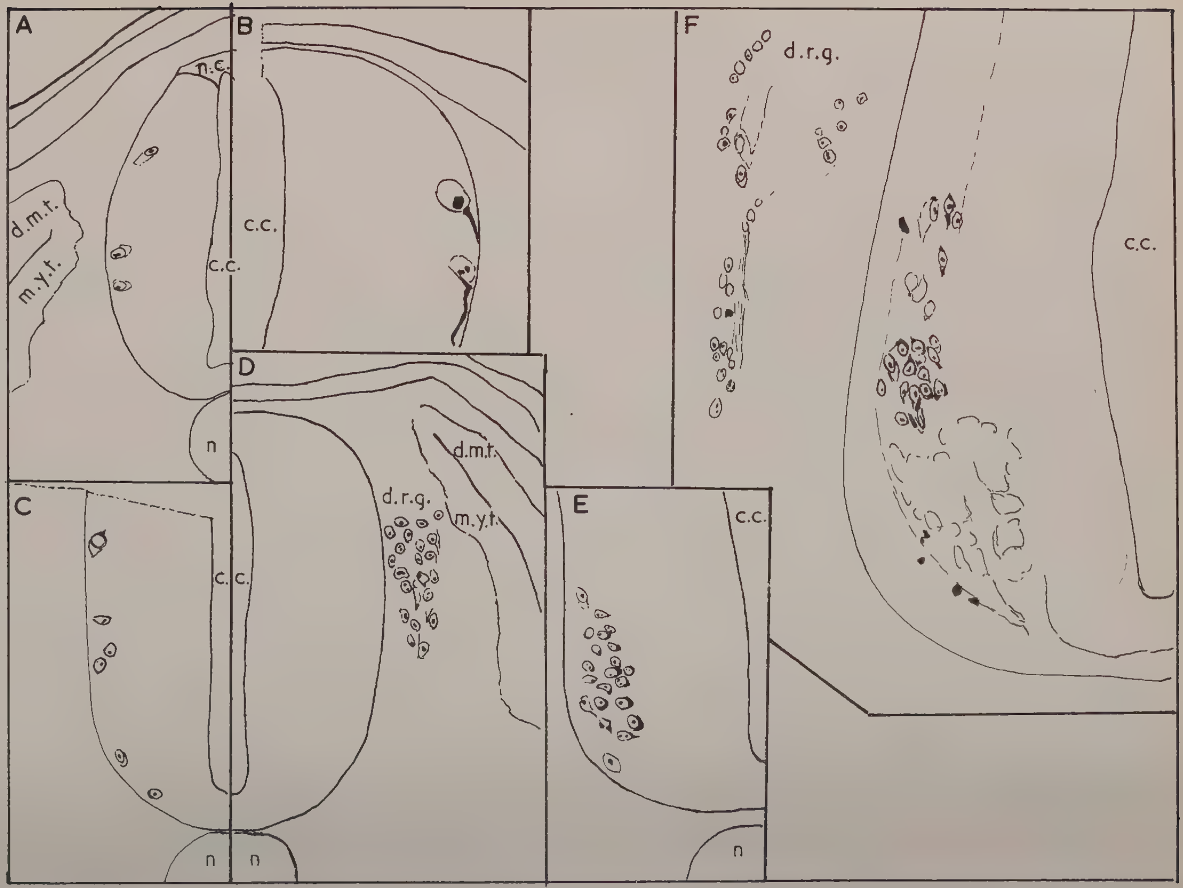
FIG. A. 50 hours. Cord at cervical level. Neuroblasts are differentiating at surface of cord without as yet any marked increase in their cytoplasmic absorption.

FIG. B. 50 hours. Silver-stained by Holmes's method to show these neuroblasts, which are becoming monopolar. ×400.

FIG. C. 58 hours. Cord at cervical level. Differentiating neuroblasts slightly more conspicuous.

FIG. D. 58 hours. Cervical level. Differentiation of neuroblasts of a dorsal root ganglion. The cells of the dermatome are denser than those of the myotome.

FIG. E. 3½ days. Brachial level. The somatic motor neuroblasts are distinguished by a dense perinuclear cap of absorbing material.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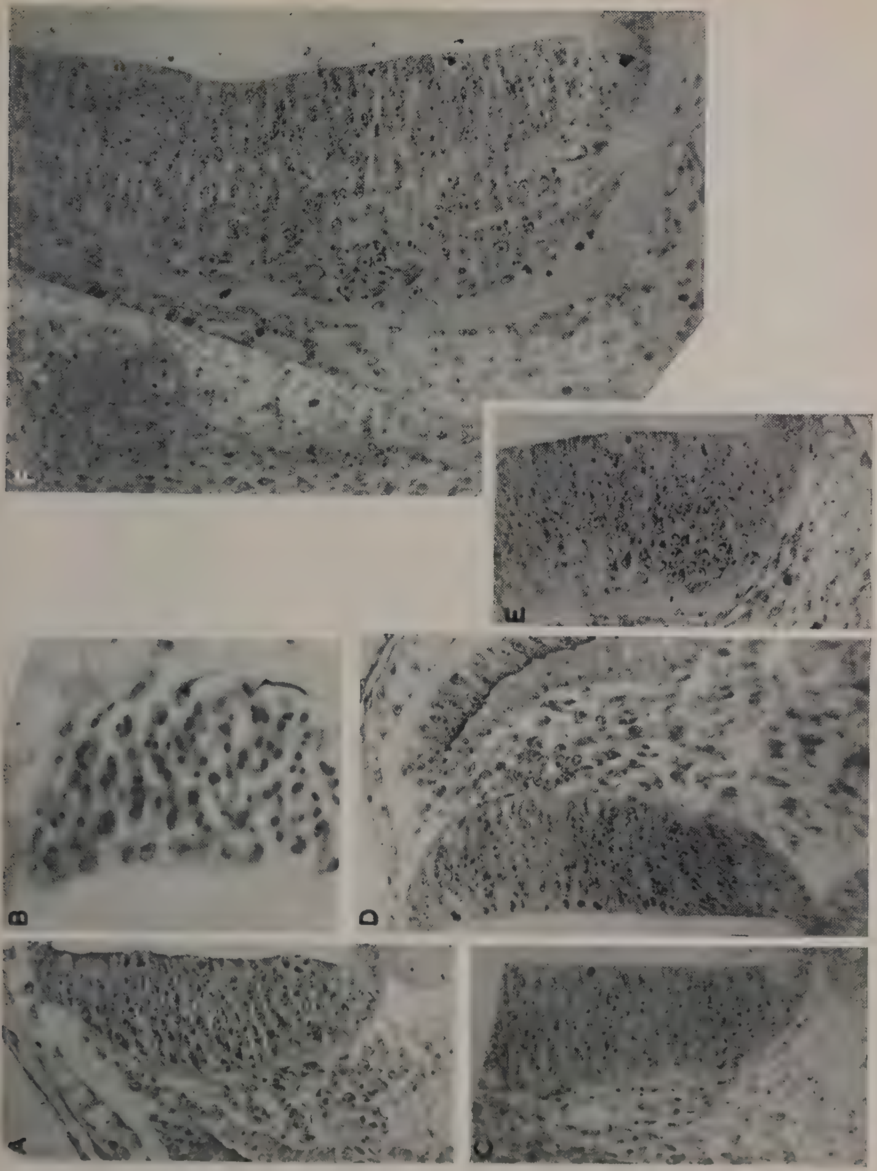
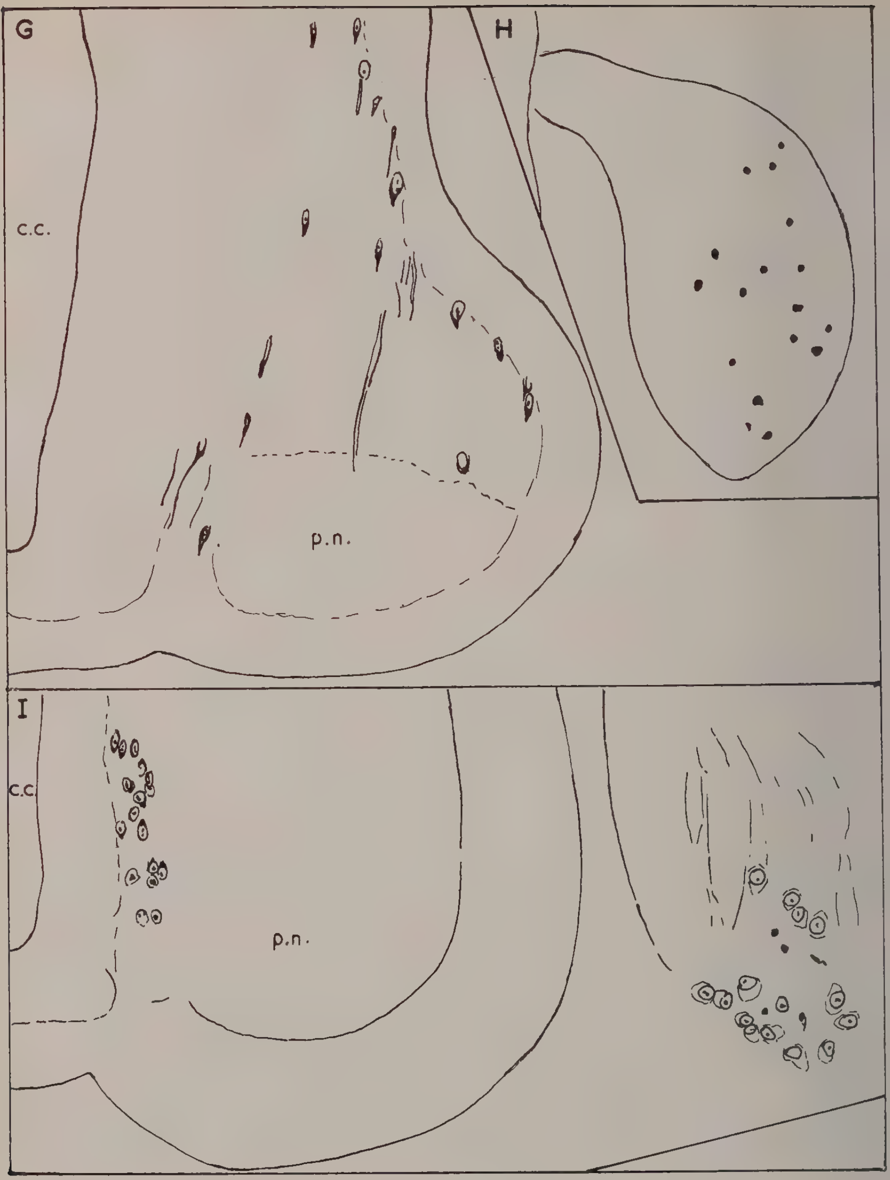


A. HUGHES

Plate 1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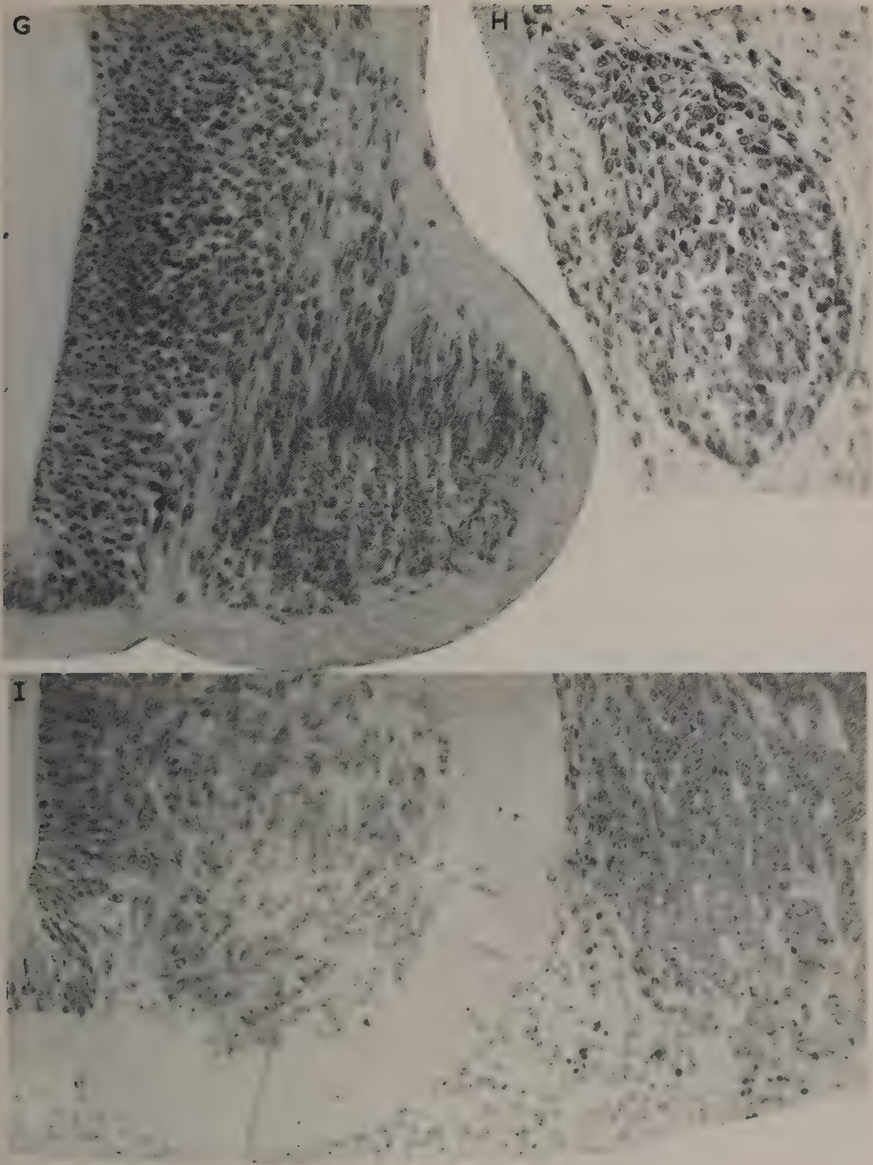
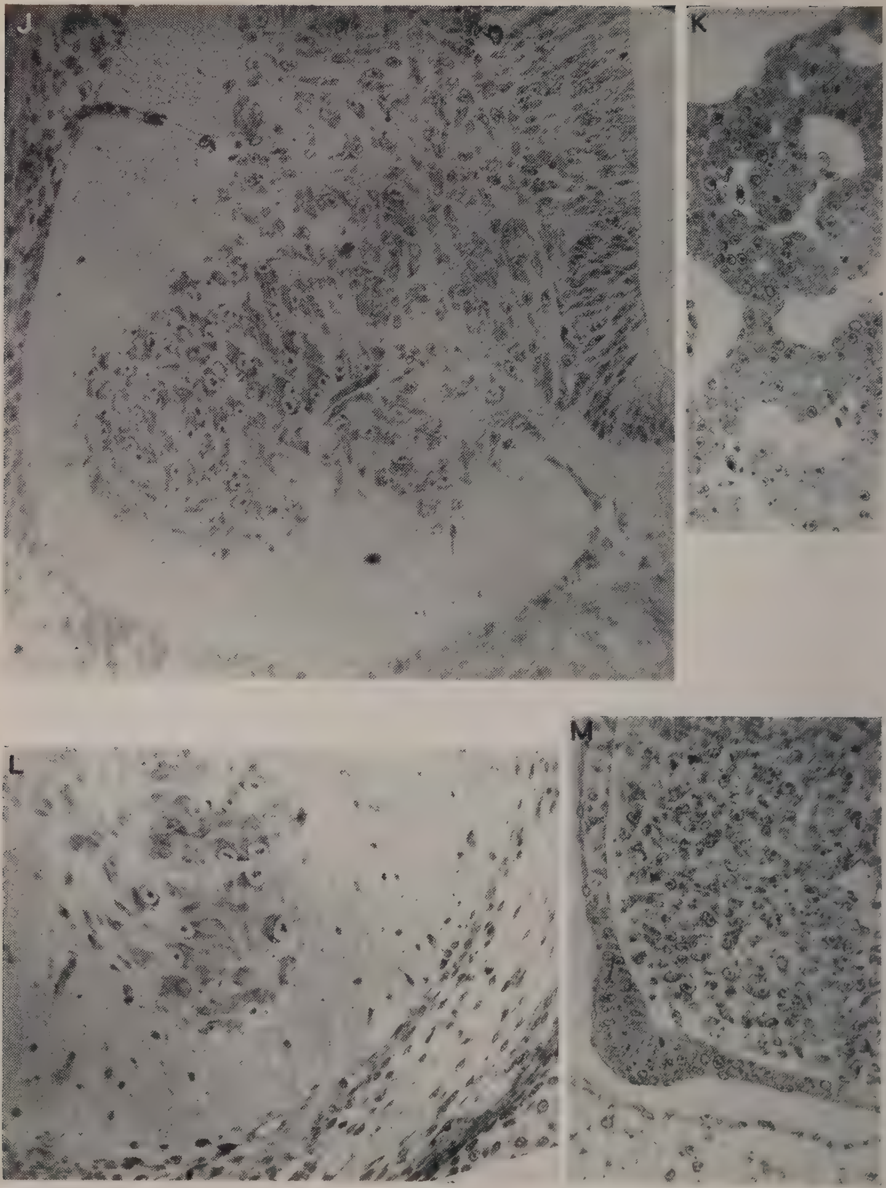


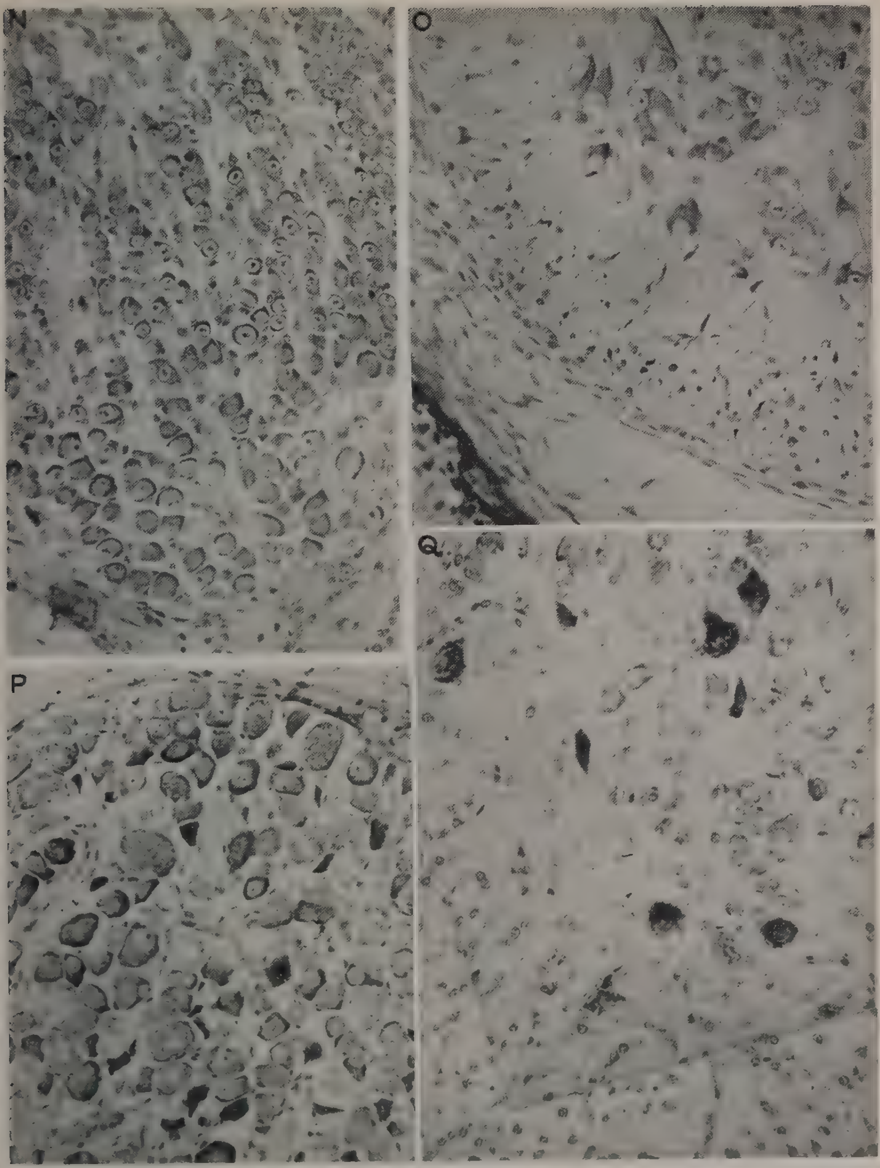
A. HUGHES

Plate 2



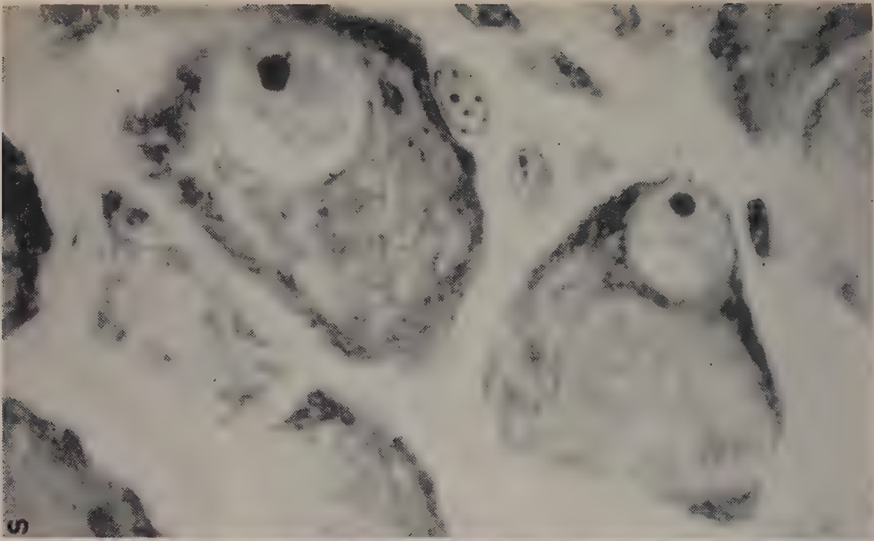
A. HUGHES

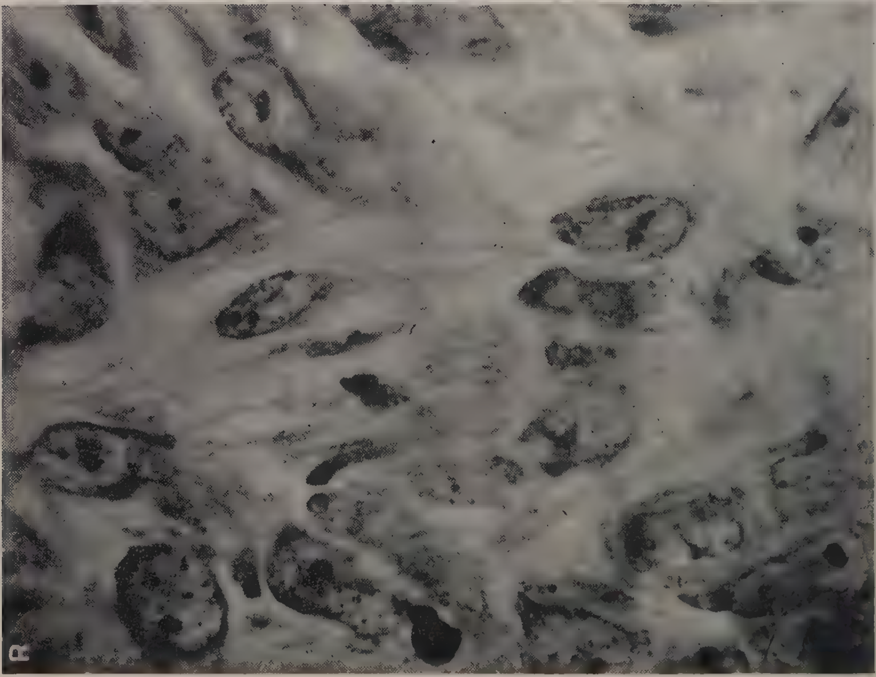
Plate 3



A. HUGHES

Plate 4





A. HUGHES

Plate 5

FIG. F. 4½ days. Thoracic level. In the cord a group of secondary neuroblasts dorsal to the primary set show dense absorption. Some pycnotic nuclei are present. The cell bodies and fibres of the ganglionic neuroblasts are conspicuous.

PLATE 2

FIG. G. 4½–5 days. Brachial level. Period of maximum general absorption. Extensive differentiation of secondary neuroblasts, mostly commissural, with distinct axons. Notice the longitudinal fibres of the white matter.

FIG. H. 4½–5 days. Cervical level. Upper portion of dorsal root ganglion. Pycnotic nuclei conspicuous.

FIG. I. 6–6½ days. Thoracic level. Period of low general absorption. Recently differentiated visceral motor neuroblasts in mantle layer. Lower part of dorsal root ganglion to right of section, showing group of relatively advanced neurones with a few pycnotic cells.

PLATE 3

FIG. J. 6½–7 days. Brachial level, ventral horn. Low general absorption. The density of the motor neurons is less than that of the blood corpuscles within a capillary in the white matter.

FIG. K. 6–6½ days. Liver. In many of the cells a sharp ring of absorbing material outlines the nuclear membrane.

FIG. L. 9 days. Ventral horn, brachial level. Part of ventral root visible. Microglial cells entering white matter.

FIG. M. 4½ days. Apical cap of limb bud. Uniform cytoplasmic absorption in proliferating cells of apical cap, and elsewhere in ectoderm and mesenchyme of limb bud.

PLATE 4

FIG. N. 9 days. Dorsal root ganglion. Thoracic level. Note that the absorption of the Nissl substance in neurons is denser than in the cells of the dorsal root ganglion (Fig. L).

FIG. O. 11 days. Ventral horn, brachial level. Density of Nissl substance in motor neurons still below that in dorsal root ganglion. Microglial cells still confined to white matter.

FIG. P. 21 days. Dorsal root ganglion, brachial level. The nuclei of many neurons is excentrically placed. Compare with Fig. S.

FIG. Q. 21 days. Ventral horn, brachial level. Maximum density of Nissl substance in motor neurones. Mesoglial cells have entered the grey matter.

PLATE 5

FIG. R. 4½–5 days. Brachial level. Part of ventral horn at high power. Notice the granularity of the neuroblast nuclei. Commissural fibres are seen running almost dorso-ventrally. One pycnotic nucleus is on the left of the figure.

FIG. S. 21 days. Two neurones of a dorsal root ganglion. The nucleoli are large, one with an adjacent chromocentre. In the lower neurone the nucleus bulges outwards, displaced by the large 'central body'. Notice the nuclei of the satellite cells.

(Manuscript received 5:xi:54)

A New Technique for the Cultivation of the Chick Embryo *in vitro*

by D. A. T. NEW[1]

From the Department of Anatomy and Embryology, University College, London

WITH ONE PLATE

INTRODUCTION

THE inaccessibility of the chick embryo in the egg has led to the invention of a large number of *in vitro* techniques for its cultivation and study. The simplest of these techniques consists of little more than pouring the entire egg contents into a suitable container and taking precautions against excessive evaporation or bacterial infection (e.g. Assheton, 1896; Schmidt, 1937; Vollmar, 1935; Romanoff, 1943). Although these methods assist observation of gross changes occurring in the embryo or its membranes, they are of little use to the embryologist who wishes to make detailed observations or operations. It is very difficult to determine the outline of, for example, a primitive streak, whilst the blastoderm still lies on the yolk; and any operation on the blastoderm is usually followed by a slow leakage of yolk through the wound, which ruins the preparation.

The most successful method devised hitherto for explanting the blastoderm in isolation from the yolk is that of Waddington (1932). It is essentially an adaptation to chick blastoderm culture of a tissue culture technique of Fell & Robison (1929). The blastoderm is transferred to the surface of a clot of fowl plasma and embryo-extract maintained in a watch-glass in a moist chamber. In this position it is readily accessible to observation and manipulation. A modification has recently been introduced by Spratt (1947) who uses a clot stiffened by agar and containing diluted egg albumen or yolk. Except in a few details the two types of clot give the same result.

The Waddington technique has proved of great value to chick embryology. Nevertheless, it has considerable limitations. Primitive streak blastoderms usually develop only as far as the beginning of flexion and torsion—equivalent to about 30 hours' development *in ovo*. Embryos explanted at earlier stages rarely develop so long. A functional blood circulation is rarely established, and if it is death always occurs shortly afterwards; this is still the case even when the embryo has a functional circulation at the time of explantation. The rapid blastoderm expansion that occurs during the first few days *in ovo* is almost completely

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brought to a standstill after explantation to a clot surface. The embryos produced are stated to be abnormally small and slow-developing (Waddington, 1932).

The technique to be described here was designed to overcome some of these limitations. It consists essentially in supporting the explanted blastoderm on a piece of vitelline membrane stretched across a glass ring. The nutrient medium is fluid albumen. Blastoderms explanted at primitive streak stages continue expanding normally, and frequently produce large well-developed embryos with functional blood circulations. The technique is suitable for the explantation of blastoderms at all stages up to 48 hours of incubation.

METHOD

The whole explantation should be carried out under sterile conditions.

An egg at the required stage of development is opened by tapping round the broad end with the handle of a scalpel. The thick albumen is removed with blunt forceps, some of the thin albumen is poured into a container to be kept for later use, and the yolk and remaining albumen is poured into a wide dish containing sufficient Panett & Compton's saline to cover the yolk completely: 3·5 cm. is a convenient depth.

At this stage any thick albumen still adhering to the vitelline membrane must be removed with forceps. If the embryo is not already uppermost, the yolk is gently turned until the embryo is brought to that position. A watch-glass is placed in the dish ready to receive the blastoderm, and the vitelline membrane is then cut with scissors along a line passing slightly above the equator of the yolk (Text-fig. 1A). The cut edge of the membrane is seized with a pair of fine forceps in each hand and gently peeled off the surface of the yolk. With care the circle of membrane can be obtained almost completely free of yolk and with the blastoderm attached to it. The membrane is pulled through the saline to the watch-glass and placed in it with the blastoderm side uppermost. It is convenient to place the glass ring[1] on top of it at this stage as it keeps the membrane from drifting, and the watch-glass with contents can then safely be transferred to a Petri dish. The floor of the Petri dish is covered with water and cotton wool and acts, as in the clot techniques, as a moist chamber.

Subsequent stages are illustrated in Text-fig. 1 B–D. Saline is pipetted off until the upper part of the ring appears above the surface. The free edge of the vitelline membrane is folded inwards and gently pulled until the membrane forms a fairly flat surface. Care should be taken not to pull the membrane so tight that the blastoderm is distorted. Any small wrinkles left in the membrane at this stage can be ignored, as they tend to disappear shortly after the preparation has been returned to the incubator and the membrane then forms a perfectly smooth surface. The edge of the membrane can now be trimmed with scissors. All but a few drops of saline are removed from above it (the blastoderm should appear thoroughly moist but it is most important to avoid leaving a pool of saline over it). The saline under the membrane is replaced with thin albumen.

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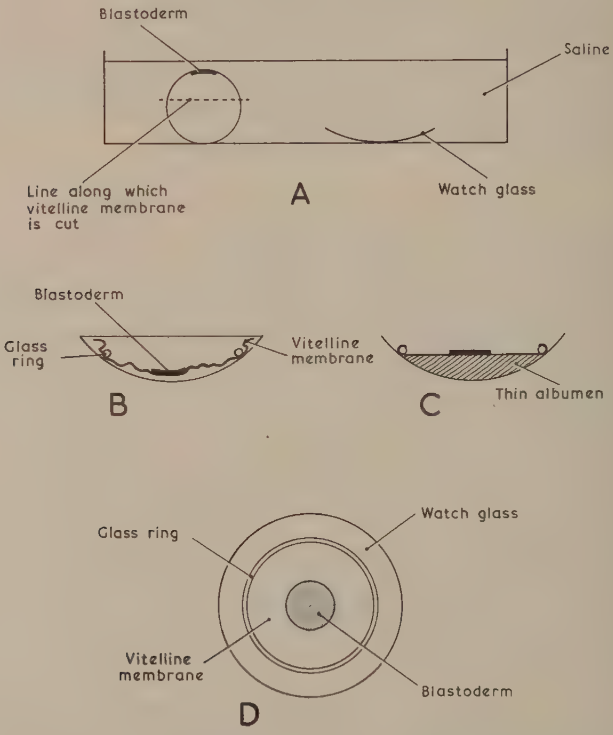
[1] Rings of internal diameter 28 mm. and external diameter 33 mm. are a useful size. Larger rings can be used, but freeing correspondingly greater areas of vitelline membrane from the yolk introduces extra technical difficulties.



TEXT-FIG. 1. A. Stage preparatory to peeling off vitelline membrane and blastoderm. B. Contents of watch-glass after removal from dish of saline. C. Completed preparation. D. Completed preparation from above.

It sometimes happens that during the manipulation part of the periphery of the blastoderm becomes loosened from the vitelline membrane. It will readily reattach itself on further incubation provided it is pressed close against the membrane. To ensure this, the membrane surface should be left as dry as possible for a few hours until attachment has taken place, and then a few drops of saline may be added.

Very young blastoderms (pre-streak or early streak stages) are only lightly attached to the vitelline membrane and tend to be left behind if the latter is peeled off the yolk. To avoid this it is better in explanting young blastoderms only to free the edges of the circle of vitelline membrane from the yolk by pulling it, and then to use a spatula to lift off the remainder together with the blastoderm and a certain amount of underlying yolk. The yolk may subsequently be gently scraped away with small knives.

RESULTS

Survival rate

The proportion of normal blastoderms that survive this form of explantation is extremely high. Out of 149 explants (mainly definitive primitive streak or head-process stages) development was found to continue in 139—a survival rate of about 93 per cent. This figure is based on a number of experiments involving eggs laid at all seasons of the year.

Extent of embryonic development

Sixty-nine explanted blastoderms (definitive primitive streak or head-process stages) were incubated until embryonic development ceased. Sixty-six of them reached at least the 10-somite stage. Fifty-three developed a functional blood circulation and forty-four went on to show pronounced flexion and torsion. After this the mortality rate increased rapidly and only fourteen reached early limb-bud stages. None developed farther.

Expansion of the blastoderm

As far as can be judged from a superficial examination, the blastoderm expands normally. Both ectoderm and endoderm spread outwards evenly until they occupy the whole area enclosed by the ring. There are no obvious abnormalities as during growth on clots (e.g. formation of cysts). After the whole available area has been covered expansion ceases; there is no tendency to form folds.

The rate of expansion also appears to be about normal. As a standard of comparison a graph was used based on the diameter of 80 blastoderms incubated *in ovo* for lengths of time ranging from 8 hours to 48 hours.

Six blastoderms explanted between 19 and 25 hours of incubation were measured after a total incubation period of 39 hours. The blastoderm diameter then had approximately the same average value (17·4 mm.) as that determined from the graph of controls *in ovo*.

In another experiment two blastoderms were found just to have filled rings of internal diameter 28 mm. after 47·5 hours' incubation (of which 23·5 hours were *in vitro*); again this agreed well with the controls.

Rate of embryonic development and size of embryo

Average values of growth rates of explanted embryos were compared with the data given by Hamburger & Hamilton (1951) for normal growth *in ovo*. The time taken for definitive primitive streak blastoderms to develop 20-somite embryos was approximately the same (about 32 hours) *in vitro* as *in ovo*. After the 20-somite stage development *in vitro* became progressively slower than *in ovo* and ceased altogether at about 30 somites.

Insufficient measurements have been made to give a detailed comparison between growth in size of embryos explanted by this technique as compared with those *in ovo*. However, blastoderms explanted at primitive streak stages usually give 12-somite embryos about 6 mm. long, and 30-somite embryos about 9–10 mm. long, which appears to be approximately normal.

Amnion development

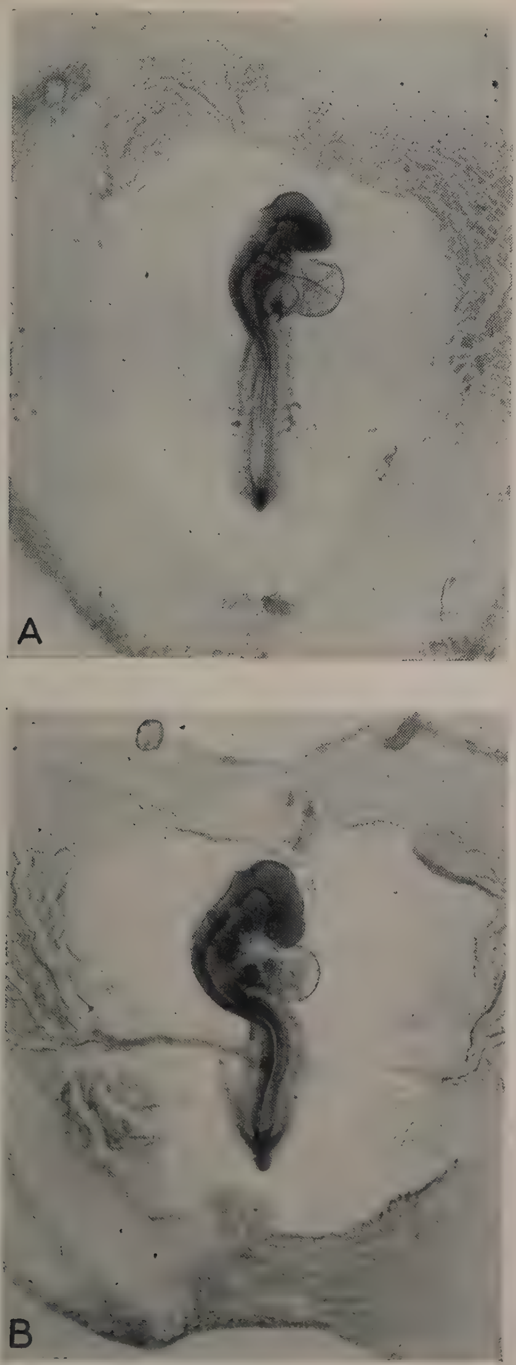
The development of the amnion seems to be markedly affected by explantation. It has frequently been observed that the amnion is less developed than expected for an embryo of a given stage. Several embryos that developed as far as limb bud formation were found to have no trace of an amnion although, according to Hamburger & Hamilton, it should by this stage almost have covered the embryo. Sometimes, however, the amnion does partially develop; in some explants it has been found covering about half the embryo.

DISCUSSION

The results indicate that this method of blastoderm explantation has considerable advantages over the previous methods involving explantation on to clot surfaces. The survival rate is higher and the amount of embryonic development that can be expected is greater. Thus primitive streak blastoderms frequently develop embryos with functional blood vascular systems and showing flexion and torsion—a rare occurrence on clot surfaces. The blastoderm expands normally until the end of the second day of incubation, and, judging by the growth rate and size of the embryo, the path of embryonic development is probably nearer the normal in these explants than in those on clots.

However, although a much higher proportion of embryos develop to advanced stages by this method as compared with clot techniques, the most advanced stage of development that can be reached appears to be about the same in both cases. Death inevitably occurs shortly after the start of limb-bud formation. Hughes (1935) suggests that the blood-vessels of embryos on clot surfaces become blocked because multiplication of corpuscles takes place at the normal rate, whilst the vessels themselves fail to increase in size. Possibly similar events occur in blastoderms growing on vitelline membranes, but it seems likely that other mechanisms also contribute towards the death of the embryo. The inverted position of the embryos may produce serious mechanical interferences with development after a certain stage; certainly this seems a likely explanation for the abnormal amnion development. The pool of watery fluid that accumulates above the blastoderm after 2 days of incubation *in vitro* may interfere with respiratory exchanges between the area vasculosa and the atmosphere; Spratt (1950) has shown that early chick development is very sensitive to any restriction in oxygen supply. Or it may be that the nutrient medium used is inadequate to support development beyond a certain stage.

The explantation technique that has been described here appears to be well



D. A. T. NEW
Plate 1

suited to studies of early chick embryology involving micro-operations. It has also been used to investigate the mechanism of blastoderm expansion and the transfer of fluids through the blastoderm. These two subjects will be discussed in subsequent publications.

SUMMARY

1. A new technique is described for the cultivation of chick embryos *in vitro*. It consists essentially in supporting the explanted blastoderm on a piece of vitelline membrane stretched across a glass ring. The nutrient medium is fluid albumen.

2. Primitive streak blastoderms explanted by this technique: (*a*) frequently develop as far as the formation of a functional blood circulation, together with flexion and torsion of the embryo; (*b*) show normal expansion across the vitelline membrane; (*c*) develop at a normal rate until after the 20-somite stage.

ACKNOWLEDGEMENTS

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EXPLANATION OF PLATE

A. Blastoderm explanted at head-process stage. Incubated 43 hours *in vitro*.

B. Blastoderm explanted at definitive-primitive-streak stage. Incubated 43 hours *in vitro*.

(Manuscript received 14 : xii : 54)

The Site of Implantation of Ova in the Rat

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INTRODUCTION

It is stated that in polytocous mammals the foetuses are implanted evenly down the uterine horns (Boyd & Hamilton, 1949). If rats are killed late in pregnancy, it is certainly found that the foetuses are evenly distributed along the length of the horns. In the course of other work, however, routine laparotomies were performed upon a large number of females 9 days after mating, and the findings apparently failed to agree with the usual belief. In consequence further laparotomies were performed on other females and a careful note was made as to the evenness or otherwise of implantation sites along the horns. The results of this survey, which are given here, showed that implantation was certainly not always as hitherto described.

MATERIALS AND METHODS

The rats used were of the Wistar albino strain as developed at the Glaxo Laboratories, Greenford, Middlesex. Mating was determined by the finding of spermatozoa in the uterine smear, and laparotomy was performed 9 days later. Note was made in every case of the number of embryos in each horn, and in which half of the horn, cranial or caudal, these were placed, as well as of sites from which conceptuses had already been resorbed (Frazer, 1955).

RESULTS

In a series of 109 females, the number of ova implanted per mother varied from 4 to 18 (apart from cases where the rats proved non-pregnant). Where there were more than 5 embryos per horn, these tended to be spaced evenly; but with smaller numbers the embryos were nearly all in the caudal halves of the horns. These results are summarized in the Table. Of the 66 horns where less than 5 ova were implanted, no ova at all were implanted in the cranial halves of 22; while in only 2 of the remaining 44 were there more implantation sites in the cranial than in the caudal halves of the horns.

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TABLE

Summary of implantation sites, according to number of ova in the horn. The top half of the table represents the findings in all the rats concerned, while the lower half gives the findings in those rats where different numbers of ova were found in the two horns of each rat

	Number of ova implanted				
Number of horns	*Per horn*	*Caudal half*	*Cranial half*	χ^2	p
66	4 or less	144	73	12·20	< 0·001
151	5 or more	533	466		
54	4 or less	113	63	5·69	< 0·02
54	5 or more	186·5	163·5		

DISCUSSION

These results show that implantation with the ova evenly spaced along the horn tended to occur if the total number of these ova was high; but that with lower numbers the ova tended to implant in the caudal halves of the horns. The fact that towards term the foetuses are evenly spaced suggests that there is either a variable growth rate in different parts of the horn (which must be influenced by the local presence or absence of conceptuses), or else that litters with more caudal implantation must be totally lost some time after the 9th day; so that the only mothers reaching examination at term are those who originally had even spacing of the implanted ova. It has already been shown (Frazer, 1955) that there is an increased rate of loss of whole litters after the 9th day where less than 9 ova were implanted. The fact that not all such litters are lost suggests that both factors must normally be concerned in producing the final even spacing of foetuses along the horn.

The mechanism controlling the sites of implantation must also be sought. Passage of the ova along the horns is brought about as a result of uterine movements, and these have been shown in the rat (Blair, 1923) to vary with the stage of the sexual cycle, and hence with the output of the ovarian hormones. It might be argued that the position of the implantation sites in the horns was a consequence of some general hormonal effect; against this must be adduced the findings in the lower half of the table. Here are displayed the findings in 54 rats where one horn had a high number of ova implanted, and the other a low number. It is seen that there were once again twice as many foetuses in the caudal halves as in the cranial halves when low numbers were implanted; while there was no such marked difference between the halves of the other horns. Thus there was a difference between the behaviour in the pairs of horns which were in the same hormonal environment. So the numbers of ova themselves (and not any hormonal level varying with this) were responsible for the differences in the distribution of implantation sites.

SUMMARY

1. In the rat, implantation sites on the 9th day of pregnancy were only evenly distributed along the uterine horns when there were 5 or more ova in one horn.

2. With lesser numbers ova tended to implant in the caudal end of the horns.

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Malformations déterminées par des injections de substances chimiques localisées à la tête d'embryons de poule

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AVEC TROIS PLANCHES

NOUS avons cherché à montrer dans des travaux antérieurs (Ancel, 1954) sur l'embryon de Poule, l'utilité des injections localisées en chimiotératogenèse, en particulier pour la réalisation de malformations n'existant pas à l'état spontané chez les Oiseaux comme la symélie ou rares comme l'ectrosomie. La réalisation des malformations de la tête qui fait l'objet de ce mémoire s'ajoute à ce sujet à nos résultats antérieurs. Les injections localisées à la tête d'embryons de Leghorn blanches ont été faites à l'aide d'un appareil spécial (Ancel & Houget, 1954). Elles ont été réalisées dans les conditions suivantes.

CONDITIONS EXPÉRIMENTALES ET MALFORMATIONS OBTENUES

Trois séries d'expériences ont été faites sur un ensemble de 571 embryons. La première a été entreprise dans un but d'orientation; les injections ont été faites entre 26 et 34 heures d'incubation à 145 embryons. Elles variaient de 1/8 à 2/8 mm.^{3} Quatre substances ont été étudiées en solutions aqueuses à diverses concentrations: la trypaflavine de 1 : 500 à 1 : 2.000; la ricine de 1 : 10.000 à 1 : 40.000; la saponine (pure et blanche Scarlach) de 1 : 1.000 à 1 : 3.000 et la colchicine de 1 : 10.000 à 1 : 30.000.

La seconde série nous a permis de réaliser une malformation à l'état isolé (exencéphalie avec encéphale fermé) en forte proportion par injection à 99 embryons de 1/24 mm.^{3} de solution de colchicine à 1 : 30.000 après 34 heures d'incubation. La troisième série a été effectuée pour connaître les différences dans l'action tératogène des quatre substances à un stade bien déterminé (4 somites) obtenu après 26 heures d'incubation. Des injections de 1/24 mm.^{3} ont été faites à 327 embryons avec des solutions de quatre substances à des concentrations données dans le tableau 1.

Les malformations obtenues peuvent être divisées en trois groupes com-

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prenant: (1) les malformations du crâne et de l'encéphale, (2) les malformations de la face, (3) l'acéphalie.

Malformations du crâne et de l'encéphale

Elles concernent l'exencéphalie et les lésions de l'encéphale.

(*a*) *Exencéphalie*

La principale malformation obtenue dans nos expériences est l'exencéphalie; nous en avons observé deux variétés qui doivent être bien différenciées l'une de l'autre parce qu'elles se réalisent par des mécanismes différents. La première est due à un arrêt de développement de la voûte crânienne à un stade où la partie de la gouttière nerveuse qui formera l'encéphale est déjà transformée en tube: l'encéphale hernié est entouré des méninges. La seconde variété est déterminée par l'absence de fermeture de cette partie antérieure de la gouttière nerveuse; l'encéphale hernié est ouvert, ses cavités sont à nu et il est en continuité avec l'ectoderme sur sa périphérie. Ces différences entre les deux variétés nécessitent une appellation différente pour chacune d'elles.

Nous nommerons *cléiencéphales* (de κλείω je ferme) les exencéphales à encéphale fermé et *acléiencéphales* les exencéphales à gouttière nerveuse restée ouverte. Après l'exposé de nos résultats, nous donnerons les raisons pour lesquelles il nous a paru indispensable de remplacer par ces dénominations celles utilisées par les auteurs dans leurs recherches sur les malformations de l'encéphale chez l'Homme et les Vertébrés (Voir: Nomenclature, p. 344).

Dans une publication antérieure (Ancel, 1950), nous avons donné les résultats de l'examen de 5.000 embryons, au cours duquel nous n'avons pas trouvé d'exencéphales à encéphale fermé et 1,2 pour cent d'exencéphales à encéphale ouvert que nous avons appelés anencéphales comme les auteurs qui nous ont précédé, bien que l'encéphale soit bien développé.

En l'absence d'autres statistiques analogues, nous admettrons qu'en tant que malformation spontanée on rencontre l'acléiencéphalie dans une proportion un peu supérieure à 1 pour cent et la cléiencéphalie trop rarement pour influencer les résultats expérimentaux que nous allons exposer.

(1) *Cléiencéphalie.* Nous avons décrit dans un travail antérieur (Ancel, 1955) cette variété d'exencéphales que nous avons obtenue dans notre première et dans notre deuxième série d'expériences. Dans la première série, sur 51 embryons morts après le sixième jour de l'incubation 31 présentaient cette variété d'exencéphalie accompagnée de malformations de la face chez 16 d'entre eux. Chez les 15 autres, aucune de ces malformations de la face n'a été constatée (Fig. A, Planche 1), mais seulement chez quelques-uns une légère micrognathie inférieure accompagnée d'une absence de revêtement cutané sur la partie supérieure de l'œil entraînant l'absence de la paupière supérieure mais sans malformation macroscopiquement visible de l'œil (Fig. B, Planche 1).

Dans la seconde série d'expériences, sur 53 embryons morts après le sixième jour, nous avons obtenu 22 exencéphales (41,5 pour cent) 4 avec malformations de la face et 18 sans ces malformations. Parmi ces derniers, 13 sont morts entre le dixième et le vingtième jour de l'incubation et 5 ont été prélevés vivants le vingtième ou le vingt-et-unième jour au moment de l'éclosion des témoins.

Dans notre troisième série d'expériences, nous avons encore obtenu des cléiencéphales avec les quatre substances utilisées dont le nombre pour chacune d'elles se trouve dans le tableau 1.

Parmi les exencéphales obtenus dans ces trois séries d'expériences, les uns présentaient une exencéphalie limitée aux hémisphères cérébraux (proencéphalie), d'autres aux lobes bijumeaux et au cervelet (notencéphalie) et d'autres enfin aux parties de l'encéphale hernié dans ces deux premières variétés réunies (hyperencéphalie). Quelques-uns n'étaient exencéphales que d'un côté (cléiencéphalie unilatérale) ou dans une région très restreinte et quelconque de la voûte crânienne (cléiencéphalie localisée).

(2) *Acléiencéphalie.* Cette malformation n'ayant été observée que sur des embryons ayant reçu une injection avant la trente-quatrième heure de l'incubation dans notre première série d'expériences, nous avons été amené à réaliser la troisième dont nous ferons seulement état ici. 327 embryos ont été injectés après 26 heures d'incubation avec 1/24 mm.^{3} d'une solution de 4 substances aux concentrations données dans le tableau 1. Parmi ces embryons, 96 sont morts avant le troisième jour de l'incubation; la plupart étaient porteurs de très graves lésions de la tête, indéchiffrables à la loupe. Sur les 231 restants, nous avons trouvé 39 acléiencéphales, soit 12 pour cent. Le nombre en est donné pour chaque substance dans le tableau. La proportion est la suivante: ricine 5 pour cent, trypaflavine 36 pour cent, saponine 27 pour cent, colchicine 0.

La mort de ces acléiencéphales s'est produite entre le sixième et le dixième jour de l'incubation pour 25 d'entre eux (61,4 pour cent); entre le onzième et le seizième jour pour 12 (30,2 pour cent) et entre le dix-septième et le vingt-et-unième pour 2 (5,1 pour cent). La survie des acléiencéphales paraît donc plus difficile que celle des cléiencéphales.

Différents degrés dans la non fermeture de l'encéphale ont été observés. Le cerveau antérieur est souvent seul ouvert et cette ouverture peut même n'intéresser que sa partie antérieure (Fig. C, Planche 1). Dans certains cas, l'ouverture du cerveau antérieur se prolonge sur le cerveau moyen qu'elle intéresse sur une plus ou moins grande étendue. La possibilité d'une ouverture atteignant le cerveau postérieur par action de substances chimiques dans nos conditions expérimentales doit être réservée bien que nous ayons observé deux cas (un après injection de saponine et l'autre après injection de trypaflavine) dans lesquels tout l'encéphale était ouvert.

En voici les raisons: au cours de nos expériences concernant les œufs ouverts après 26 heures d'incubation, aucune injection n'a été faite aux embryons qui avaient un retard de développement suffisant pour que la tête de l'embryon ne soit pas visible, mais nous laissions ces embryons se développer, sans en tenir compte dans l'expérience. Ils étaient au nombre de 71; la plupart sont morts au troisième jour, d'autres se sont développés sans présenter de malformations tandis que sur d'autres enfin nous en avons observé et en particulier de l'acléiencéphalie chez 5 d'entre eux. Dans ces 5 cas, l'encéphale était ouvert en totalité et dans un cas la moelle cervicale l'était aussi (Fig. D, Planche 1). Ces 5 cas sur 71 embryons constituent une proportion élevée; elle s'élève si on y ajoute les deux cas litigieux (7 sur 71 soit 10 pour cent). Ce résultat n'est en réalité pas en opposition avec celui de notre statistique d'acléiencéphalie spontanée portant sur 5.000 embryons, car nous avions remarqué dans ces œufs ouverts après 48 heures d'incubation que les acléiencéphales étaient presque tous en retard de développement au moment de l'ouverture de l'œuf. Les 71 embryons non injectés étaient donc sélectionnés parmi les embryons capables de donner des acléiencéphales. La proportion de 7 acléiencéphales doit donc être calculée sur tous les embryons mis en expérience, soit 327 + 71 = 398, soit un peu plus de 1 pour cent. Le fait que nous n'avons rencontré aucun acléiencéphale sur 95 embryons injectés avec la colchicine parle en faveur de l'exactitude de ce raisonnement.

Le tableau 1 résume les principaux résultats de notre troisième série d'expériences. Il a pour objet de mettre en évidence les différences obtenues dans l'action tératogène des quatre substances étudiées. Il rapporte le nombre des embryons injectés, celui des morts avant le troisième jour dé l'incubation, celui des acléiencéphales, cléiencéphales et acéphales observés et celui des malformations de la face sans exencéphalie, ainsi que les concentrations utilisées.

TABLEAU 1

Expériences d'injections localisées à la tête d'embryons de poule après 26 heures d'incubation

Substances	*Concentrations*	*Nombre d'embryons*	*Morts 3 jours*	*Embryons injectés*	*Cléiencéphales*	*Acléiencéphales*	*Acéphales*	*Malfor. face sans exencéphalie*	% *Proportio n toxique*	% *Proportion tératogène*
Colchicine	1:10.000 à 1:40.000	95	32	63	11	0	3	7	33	33
Ricine	1:10.000 à 1:60.000	82	26	56	27	3	7	2	31	70
Saponine	1:1.000 à 1:3.000	71	16	55	9	15	0	12	22	65
Trypaflavine	1:1.000 à 1:3.000	79	22	57	6	21	0	6	28	58
		327	96	231	53	39	10	27		

Dans un assez grand nombre de cas, le cerveau antérieur était ouvert et le moyen fermé et revêtu des méninges. Ces cas d'association (acléio-cléiencéphalie) des deux malformations ont été comptés comme acléiencéphales, le fait essentiel étant pour nous de savoir si l'encéphale hernié n'était pas totalement fermé.

Dans ce tableau figurent aussi des chiffres concernant le pouvoir tératogène et la puissance toxique manifestés par chacune des quatre substances étudiées. La puissance toxique a été établie en comparant le nombre des embryons morts au troisième jour de l'incubation avec celui des embryons ayant reçu une injection. Cette puissance s'est montrée proportionnelle à la concentration. Par exemple, pour la colchicine, la proportion des morts a été de 70; 51,2; 33,3 et 14 pour cent respectivement pour les concentrations de 1 : 10.000, 1 : 20.000, 1 : 30.000, 1 : 40.000. Le pouvoir tératogène calculé en comparant le nombre des embryons porteurs d'une malformation de la tête avec celui des embryons mis en expérience n'a pas, dans ces limites, sensiblement varié avec la concentration; il a été de 11,7, 14,6, 15 et 14,3 pour cent. Avec les fortes concentrations, le nombre des morts augmente ainsi que la proportion des porteurs de malformations par rapport aux survivants et avec les faibles concentrations le nombre des survivants augmente mais aussi celui des embryons sans malformation.

(*b*) *Malformations de l'encéphale*

Etant donné le but de notre travail, nous n'avons pas jugé nécessaire de faire des coupes permettant d'étudier au microscope les lésions de l'encéphale, nous nous sommes limité aux dissections et aux coupes à la main.

Chez les cléiencéphales n'ayant pas de malformations de la face, l'encéphale ne montrait pas de lésion. Il en était souvent de même pour les acléiencéphales, même dans les cas où l'encéphale était largement ouvert, mais nous avons aussi observé sur les parties antérieures du cerveau antérieur, rejetées latéralement, des lésions d'ordinaire symétriques et minimes, bien localisées, et au niveau desquelles se produisaient des hémorragies qui se répandaient dans le liquide amniotique. Ces lésions de l'encéphale peuvent être dues à la substance tératogène, mais aussi au fait que l'encéphale n'est pas protégé par le revêtement méningé. L'existence fréquente, que nous avons antérieurement signalée, dans les cas spontanés, d'hémorragies dues à des ruptures vasculaires localisées dans la même région de l'encéphale hernié est en faveur de cette deuxième hypothèse. Dans certains cas d'acléiencéphalie expérimentale, nous avons au contraire observé des hémorragies étalées sur une grande partie de la surface du cerveau antérieur ouvert. Ces hémorragies du tissu nerveux n'ont pas été constatées chez les cléiencéphales, mais seulement des hémorragies méningées superficielles.

Chez les cléiencéphales, les malformations de l'encéphale portent surtout sur le cerveau antérieur qui n'est pas divisé en deux hémisphères mais forme une masse unique plus ou moins développée. Le cerveau antérieur et le cerveau moyen peuvent faire défaut chez les cléiencéphales porteurs de malformations de la tête, soit totalement, soit en partie. Nous avons aussi observé quelques cas dans lesquels la dure-mère faisait défaut sur une certaine étendue de l'encéphale lésé lui aussi à ce niveau. Il faisait hernie par cette ouverture méningée et apparaissait plus ou moins dissocié. L'absence de la dure-mère était, dans ces cas, localisée à une des faces latérales de l'encéphale. Aucune confusion n'est possible avec l'acléiencéphalie étant donné la localisation et l'aspect du cerveau lésé.

Le bulbe a toujours présenté son aspect normal.

Dans les cas où l'encéphale apparaît normalement développé, il peut cependant présenter une malformation déterminée par une saillie de la hernie encéphalique plus forte d'un côté que de l'autre. Cette différence est due à ce que la brèche existant dans la voûte crânienne est asymétrique. Dans ces conditions, la ligne de séparation entre les hémisphères au lieu d'être médiane est oblique et son extrémité antérieure est située du côté où la brèche est la plus large. Ce déplacement latéral de la région antérieure des hémisphères s'accompagne du déplacement dans le sens opposé de leur extrémité postérieure. Ils ont donc subi un mouvement de rotation qui n'affecte pas les autres parties de l'encéphale. Le cervelet reste médian et les lobes bijumeaux restent situés à égale distance du plan sagittal médian. Il s'ensuit que du côté où la brèche est la plus petite un espace sépare l'extrémité postérieure de l'hémisphère du lobe bijumeau du même côté tandis que de l'autre ils sont au contact l'un de l'autre. Ce déplacement limité aux hémisphères est particulièrement net dans les cas d'exencéphalie unilatérale avec microphtalmie du côté de la brèche osseuse.

Malformations de la fàce

Ces malformations accompagnent habituellement l'exencéphalie mais elles peuvent aussi exister sans exencéphalie.

(*a*) *Malformations de la face sans exencéphalie*

Les embryons porteurs de ces malformations étaient tous anophtalmes ou microphtalmes, unilatéraux ou bilatéraux. Cette malformation oculaire était dans un cas associée à une très forte réduction de toute la tête après action de la colchicine et dans un autre à une micrognathie supérieure après action de la saponine. Le nombre des cas observés pour chacune des 4 substances expérimentées est donné dans le tableau. La plus forte proportion a été obtenue avec la saponine (12 pour cent).

(*b*) *Malformations de la face coexistant avec l'exencéphalie*

Le type le plus souvent observé de ces malformations associées possédait les caractères suivants: les yeux sont absents ou représentés par deux petites vésicules parfois légèrement pigmentées. Cette anophtalmie ou microphtalmie très accentuée s'accompagne d'une très forte atrophie des bourgeons maxillaires supérieurs. Chacun d'eux apparaît comme un petit diverticule du maxillaire inférieur pourvu d'un petit prolongement transversal représentant l'apophyse palatine. Entre ces apophyses atrophiées, la fente palatine est très large, surtout en avant. Le demi-bec supérieur fait totalement défaut, aucune des parties qui le constituent normalement (bourgeon frontal (B.F.) et bourgeons maxillaires supérieurs (B.M.S.)) ne s'étant développée. Le demi-bec inférieur et la langue ont au contraire leur développement normal. La figure E représente un de ces cas typiques. On y voit la hernie encéphalique faisant saillie au-dessus de la langue et bordée latéralement par les B.M.S. atrophiés. En arrière d'eux, on aperçoit le trou auditif. La dissection montre que le bord de la brèche osseuse par laquelle sort l'encéphale est formé dans sa partie postérieure par l'occipital sur lequel s'insère la partie la plus antérieure des muscles superficiels de la nuque.

Nous avons observé d'autres variétés de malformations de la face associées à l'exencéphalie. Elles diffèrent du type décrit soit par la diminution, soit par l'augmentation de ces malformations.

Dans le premier des deux groupes ainsi constitués, nous avons trouvé les variétés suivantes qui constituent une série de degrés tendant vers le type normal. La première variété s'en rapproche par le développement plus ou moins complet du bourgeon frontal. Il constitue une petite tige dont l'extrémité libre apparaît sous la hernie encéphalique (Fig. F, Planche 1) ou une tige renflée à son extrémité libre en deux saillies formées par les prémaxillaires (Fig. G, Planche 2). Les bourgeons maxillaires supérieurs (B.M.S.) ne sont pas en contact avec cette tige. Dans d'autres cas, le bourgeon frontal plus ou moins développé est soudé d'un côté seulement au B.M.S., il est alors dévié du côté soudé (Fig. H, Planche 2).

Dans une deuxième variété, l'anophtalmie ou la microphtalmie persiste mais les B.M.S. se sont développés et soudés au bourgeon frontal; le demi-bec supérieur est court (micrognathie supérieure) et droit (Fig. I, Planche 2, chez un cléiencéphale et Fig. J, Planche 2 chez un acléiencéphale) ou recourbé (Fig. K, Planche 2, dans un cas de microphtalmie unilatérale) ou dévié latéralement (Fig. L, Planche 2, chez un acléiencéphale).

Une troisième variété est caractérisée par l'anophtalmie, la microphtalmie ou la buphtalmie accompagnant la micrognathie supérieure (Fig. M, Planche 3).

Une quatrième variété sert de transition entre la précédente et l'exencéphalie pure à laquelle elle adjoint une légère micrognathie supérieure et l'absence d'une paupière (Fig. B, Planche 1).

Les variétés du second groupe peuvent être divisées en deux. La première est caractérisée par l'absence complète des yeux et du demi-bec supérieur accompagnée d'une déficience du demi-bec inférieur. La face n'est ainsi plus représentée que par le demi-bec inférieur plus ou moins rudimentaire. Dans certains cas, il a encore un assez grand développement mais il est fortement relevé vers la hernie encéphalique et plus ou moins dévié d'un côté (Fig. N, Planche 3); dans d'autres, il n'est représenté que par un petit diverticule (Fig. O, Planche 3) qui peut lui-même faire défaut. L'embryon ne diffère plus des acéphales extérieurement que par la présence des conduits auditifs.

Acéphalie

Nous rangeons sous cette appellation les cas dans lesquels la section réalisée par la décapitation chimique passe en arrière des orifices auditifs. Sa direction avoisine l'horizontale, une petite partie du demi-bec inférieur et de la langue peut, dans certains cas, persister (Fig. P, Planche 3) mais tout l'encéphale fait défaut.

Sur les 571 embryons mis en expériences, nous avons trouvé 18 acéphales; deux des substances expérimentées sur quatre ne nous ont donné aucun cas d'acéphalie (trypaflavine et saponine). La ricine en a fourni 13 cas et la colchicine en a donné 5. L'acéphale le plus âgé obtenu avec la colchicine est mort à 11 jours et à 19 jours avec la ricine. Le dixième jour de l'incubation n'a été dépassé que par un acéphale obtenu à l'aide de la colchicine et trois après action de la ricine (11–18–19 jours). Tous ces acéphales étaient porteurs de la malformation typique donnée par la colchicine et la ricine (celosomie).

DISCUSSION

Nous envisagerons successivement différents problèmes à la solution desquels nos résultats peuvent apporter une contribution.

Sensibilité élective d'une ébauche organique

Les recherches faites en collaboration avec Mme Lallemand et dans lesquelles nous déposions sur l'embryon après 48 heures d'incubation une goutte (1/20 c.c.) de solution de diverses substances chimiques (Ancel, 1950) ont mis en évidence que la malformation obtenue dépendait non seulement du stade auquel avait lieu l'intervention mais aussi de la nature de la substance injectée. Les injections localisées à la tête de l'embryon apportent, comme les injections générales, des faits intéressant l'influence du stade, de la substance injectée et de la constitution de l'embryon sur les variétés de malformations obtenues. Nous nous placerons successivement à ces trois points de vue.

(*a*) *Influence du stade*

Les injections de nos quatre substances faites après la trente-quatrième heure de l'incubation n'ont pas produit l'acléiencéphalie tandis que nous avons obtenu cette malformation quand les injections étaient faites à la vingt-sixième.

(*b*) *Influence de la nature de la substance*

L'acléiencéphalie n'a pas été obtenue avec les quatre substances expérimentées après 26 heures d'incubation. Tandis que la trypaflavine et la saponine ont donné des acléiencéphales en forte proportion (36 et 27 pour cent), la ricine n'en a donné que rarement (5 pour cent) et la colchicine aucun.

(*c*) *Influence de la constitution individuelle des embryons*

Au cours de nos expériences d'injections localisées à la tête d'embryons après 26 heures d'incubation, nous avons rencontré trois malformations différentes à l'état isolé: la microphtalmie, la cléiencéphalie et l'acléiencéphalie. Au contraire, les malformations du demi-bec supérieur n'ont pas été observées sans exencéphalie habituellement accompagnée de microphtalmie et celles de la langue et du maxillaire inférieur sans malformation du demi-bec supérieur.

Les différences observées suivant les embryons dans la mise en évidence de l'ébauche de la tête la plus sensible ne sont pas dues à des différences de stade même minimes ni à la technique utilisée. Les injections ont en effet été faites à un stade bien déterminé et quelque faible qu'ait été la quantité de solution injectée, les ébauches des yeux, celle de la partie antérieure de la gouttière nerveuse et celle de la voûte du crâne sont trop voisines pour ne pas avoir reçu chez un même embryon la même dose de substance tératogène. Il est en particulier facile de constater, au moment de l'injection faite à 26 heures, que le tube nerveux n'est pas fermé et de localiser l'injection avec une grande précision quand l'embryon est au stade recherché. Cependant, certains d'entre eux deviennent cléiencéphales, d'autres acléiencéphales et d'autres microphtalmes.

Les injections localisées permettent donc de montrer que les différences individuelles au point de vue de la sensibilité des embryons aux substances chimiques tératogènes ne sont pas des différences dans le degré de sensibilité de tout l'organisme mais dans celle de certaines ébauches seulement. L'hypersensibilité d'une ébauche à une substance chimique pouvant coexister chez le même embryon avec l'hyposensibilité d'une autre ébauche à cette même substance et inversement chez un autre embryon.

Les malformations indirectes

Nous examinerons successivement les malformations du corps qui pourraient être dues aux malformations de la tête et celles de la tête qui pourraient être conditionnées par celles de l'encéphale.

(*a*) *Malformations indirectes du corps*

La seule malformation du corps que nous ayons vu coexister avec l'exencéphalie est la célosomie. Son existence s'explique par le passage dans le sang des substances utilisées, leur injection dans le réseau de l'aire vasculaire la produisant fréquemment sans aucune malformation de la tête.

Aucune répercussion macroscopiquement visible des malformations de la tête sur le développement des ébauches du corps n'a en somme été constatée. Ce développement du corps peut être identique à celui des témoins quelque graves que soient les lésions de la face accompagnant l'exencéphalie et même dans les cas d'acéphalie. Ce résultat confirme ceux qu'ont obtenu Wolff & Stoll (1937), Stoll (1939), et Kantor (1937) après obtention d'arhinencéphales, de cyclocéphales et de triocéphales à la suite d'irradiations localisées à la tête de jeunes embryons de Poule.

Mais si les lésions de la tête n'entraînent pas de malformations secondaires du corps, il n'en est pas de même de l'exencéphalie sur le développement de la tête.

(*b*) *Malformations de la tête déterminées par l'exencéphalie*

Lorsque l'exencéphalie est accompagnée de fortes lésions de la face (anophtalmie ou microphtalmie et absence du demi-bec supérieur), l'encéphale plus ou moins diminué de volume est projeté en avant et en bas dans la brèche due à l'arrêt de développement de la face. L'anneau occipital, au lieu d'avoir une direction oblique voisine de l'horizontale, se rapproche de la verticale grâce à une courbure de la colonne vertébrale accompagnant une réduction de la fosse cérébelleuse, le cervelet étant lui aussi entraîné en avant. Les modifications produites dans le crâne en accord avec ce mouvement peuvent être chiffrées comme suit: chez un poussin normal au jour de l'éclosion, si l'on unit le trou auditif A d'une part au point O marquant sur la ligne sagittale médian le point le plus élevé de l'anneau occipital sur lequel s'insèrent les muscles superficiels de la nuque recouverts par la peau et d'autre part à la pointe du demi-bec inférieur B, on forme un angle OAB de 140° environ. Chez les exencéphales ne présentant pas de malformation de la face autre que celle des yeux, l'ouverture de cet angle n'est pas modifiée; elle est au contraire ramenée â 90° et même moins dans les cas où l'exencéphalie est accompagnée de ces lésions (comparer les figures B et E, Planche 1).

Organes médians doubles

Dans certains cas d'acléiencéphalie dans lesquels l'encéphale est largement ouvert en avant, le demi-bec supérieur est divisé longitudinalement en deux parties rejetées latéralement de manière à se trouver sur un même ligne horizontale et perpendiculaire au plan de symétrie bilatérale (Fig. Q, Planche 3). Le bourgeon frontal qui forme la partie médiane de ce demi-bec est divisé en deux moitiés latérales à chacune desquelles se trouve accolé extérieurement le bourgeon maxillaire supérieur. Sur chaque moitié de ce demi-bec se trouve une crête paraissant représenter une partie du dédoublement de la crête normale.

Dans d'autres cas, l'encéphale est fermé mais on trouve deux crêtes symétriques et placées côte à côte (Fig. R, Planche 3). L'absence de fusion des deux parties latérales de la tête entre elles paraît n'avoir porté que sur la région cutanée qui revêt le demi-bec supérieur tandis qu'elle porte sur tout le demi-bec supérieur dans le cas de l'acléiencéphale de la fig. Q, Planche 3. Ce cas nous apparaît ainsi comme un exemple de l'origine paire d'un organe médian.

Nomenclature

Dans son traité de tératologie, Isodore Geoffroy St. Hilaire (1832), se basant sur les recherches de son père Étienne et les siennes sur l'embryon humain, a donné une classification des monstres dont la malformation est essentiellement caractérisée par la présence d'une brèche dans la voûte du crâne. Il a divisé ces monstres en trois genres: anencéphaliens, caractérisés par l'absence complète d'encéphale, exencéphaliens chez lesquels l'encéphale fait saillie au-dessus de la tête et pseudencéphaliens, dont la substance nerveuse de l'encéphale est remplacée par une masse conjonctivo-vasculaire. Les anencéphaliens et les exencéphaliens constituent des types entre lesquels se rencontrent des intermédiaires.

Quant à la pseudencéphalie, elle peut être plus ou moins développée et contribue à augmenter le nombre des variétés des deux types précédents. Schwalbe (1909), dans son livre sur les malformations, reproduit la classification de St. Hilaire et est aussi d'avis que l'encéphale fait totalement défaut chez les anencéphales.

Ultérieurement, une étude plus complète des anencéphaliens humains a montré qu'il persiste toujours chez eux au moins une partie du cerveau postérieur. Le terme d'anencéphale n'en a pas moins été conservé et opposé à celui d'exencéphale et de pseudencéphale. Mais comme seuls des degrés dans la saillie de l'encéphale et dans l'étendue de la brèche osseuse séparaient ces différents types, on conçoit que les auteurs n'aient pas tous utilisé le même terme pour désigner la même malformation. D'autres appellations sont ainsi apparues: craniochisis, encéphalocèle, méningocèle, dysencéphalie extra-crânienne, tandis que des hypothèses étaient formulées au sujet de la pathogénie de l'anencéphalie.

I. G. St. Hilaire pensait que cette malformation, comme la plupart des autres, était due à des brides formées par des adhérences entre le foetus et ses enveloppes et Dareste (1891) a défendu que l'anencéphalie résultait d'une adhérence entre le capuchon céphalique de l'amnios et le foetus, empêchant la fermeture de la gouttière nerveuse. D'autres auteurs (Wirchow, Rabaud, 1905) ont apporté des arguments pour soutenir l'hypothèse d'une lésion produite par une méningite ou méningo-encéphalite. D'autres enfin ont avancé que l'anencéphalie était due à une agénésie empêchant la transformation de la plaque nerveuse en gouttière et en tube (Von Baer, Bischoff). Le bien-fondé de cette hypothèse a été établi par des recherches qui ont montré que la persistance de la plaque nerveuse (platyneurie) est le stade initial de l'anencéphalie. La caractéristique de l'anencéphalie apparaît donc consister dans la non-fermeture de l'encéphale et pas dans son absence plus ou moins complète puisque celle-ci peut se réaliser à un stade où le tube nerveux est définitivement fermé dans la région de la tête. Il est donc indiqué de remplacer le terme d'anencéphale par celui d'acléiencéphale.

Quant à la dénomination d'exencéphalie, elle se base pour tous les auteurs à la suite de St. Hilaire uniquement sur la saillie de l'encéphale au-dessus de la tête, en passant par une brèche dans la voûte du crâne.

Chez les Oiseaux, on rencontre à l'état spontané des monstres à encéphale ouvert comme chez le foetus humain, mais contrairement à ces derniers l'encéphale ouvert et non recouvert des méninges a un développement voisin de la normale et fait une très forte saillie au-dessus de la tête. Ces monstres ne méritent donc pas le nom d'anencéphales; ce sont des exencéphales comme ceux dont l'encéphale hernié est fermé et entouré des méninges. Le mécanisme de leur formation est le même que chez l'embryon humain mais chez ce dernier la non fermeture de l'encéphale a deux effets: d'une part, l'absence d'un mouvement morphogénétique transformant la plaque nerveuse en tube, et d'autre part l'arrêt de développement morphologique de l'encéphale. Le premier effet se retrouve chez les Oiseaux et l'encéphale reste ouvert, mais le second effet, très accentué chez l'Homme, est faible ou nul chez eux et l'encéphale ouvert prend un développement normal.

Il nous est ainsi apparu que les termes d'anencéphale et d'exencéphale, utilisés chez l'Homme, ne pouvaient permettre de distinguer les deux types d'exencéphales des Oiseaux l'un de l'autre et qu'il était nécessaire de créer dans ce but deux appellations nouvelles basées sur le caractère fondamental dont la présence ou l'absence les caractérise. Les termes utilisés par les auteurs ne peuvent en effet servir qu'à caractériser différentes variétés d'une même malformation parce qu'ils sont basés sur des caractères secondaires pouvant varier suivant les individus, les espèces et les classes (absence ou saillie plus ou moins forte de la hernie encéphalique; absence ou malformation d'une partie plus ou moins étendue de l'encéphale; développement plus ou moins grand d'une masse conjonctivo-vasculaire remplaçant le tissu nerveux). Or, la cléiencéphalie et l'acléiencéphalie ne sont pas deux degrés d'une même malformation, mais deux malformations dont le caractère commun est une brèche dans la voûte du crâne et cela aussi bien chez les Oiseaux et les Mammifères que chez le foetus humain.

Ces malformations se réalisent en effet: (1) par des mécanismes différents (arrêt de développement morphogénétique de l'ébauche nerveuse pour l'acléiencéphalie et de la voûte du crâne pour la cléiencéphalie); (2) à des stades différents (l'acléiencéphalie ne pouvant être obtenue qu'avant la fermeture de la gouttière nerveuse tandis que la cléiencéphalie peut l'être après cette fermeture); (3) avec des fréquences différentes suivant les agents tératogènes utilisés (par exemple la colchicine injectée à 63 embryons âgés de 26 heures a donné 11 cléiencéphales (17 pour cent) et aucun acléiencéphale, et la trypaflavine dans les mêmes conditions 6 cléiencéphales sur 57 (10 pour cent) et 21 acléiencéphales (36 pour cent).

Réalisation expérimentale de l'exencéphalie par les auteurs

Des malformations crânio-encéphaliques ont été produites par différents auteurs à l'aide de divers agents tératogènes chez les Oiseaux et chez les Mammifères. La plupart d'entre eux n'ont pas distingué les cas dans lesquels la fermeture de l'encéphale n'était pas réalisée de ceux où elle l'était et ont utilisé des dénominations ne permettant pas d'établir cette séparation. Un rapide exposé de leurs résultats nous servira pour justifier la nécessité de remplacer les appellations qu'ils emploient par les deux termes de cléiencéphales et d'acléiencéphales.

(*a*) *L'exencéphalie chez les Oiseaux*

Les anciens auteurs qui ont obtenu des embryons porteurs de malformations après action d'agents physiques, comme Dareste (1892), ou chimiques, comme Féré (1893–1901), signalent des anencéphales et des exencéphales mais ne les décrivent pas et n'en donnent pas de statistique permettant de savoir s'ils ont été réellement conditionnés par les agents tératogènes utilisés.

Wolff (1936) a réalisé de nombreuses malformations en concentrant un faisceau de rayons X sur des points divers d'embryons de poule, et en particulier des malformations de la face mais sans exencéphalie. 'Des cas d'anencéphalie, dit l'auteur, ont été souvent obtenus, non point avec régularité ni à la suite d'une intervention précise. Il est impossible de dire s'ils étaient dus à l'intervention directe ou si la malformation n'était pas provoquée par un facteur indirect, la dessiccation agissant au cours de ces expériences.'

En collaboration avec Mme Lallemand au cours de recherches sur l'action tératogène de la colchicine consistant en dépôt de 1/20 c.c. sur l'embryon de poules de race Sussex après 48 heures d'incubation, nous avons observé deux cas de cléiencéphalie accompagnés de micrognathie supérieure chez des embryons strophosomes. La photographie de l'un de ces embryons a été donnée par Mme Lallemand (1939). Nous n'avons jamais rencontré une semblable malformation chez des témoins ou après injection d'autres substances chimiques, mais j'ai trouvé ultérieurement chez des Leghorn deux cas de cléiencéphalie spontanée accompagnés aussi de micrognathie supérieure. L'action de la colchicine restait donc incertaine.

Gallera (1951) a fait incuber des œufs de poule dans un grand cylindre hermétiquement fermé qui contenait soit de l'oxygène, soit de l'azote, soit de ces deux gaz mélangés en proportions variées, soit encore un mélange d'oxygène et d'anhydride carbonique. La durée d'incubation dans cette atmosphère ne dépassait jamais cinq heures. Le séjour dans l'atmosphère pauvre en oxygène a donné comme malformations: la platyneurie, l'omphalocéphalie, la diplocardie et d'autres malformations du cœur. Dans une atmosphère renfermant 20 à 30 pour cent de CO^2, même si la quantité d'oxygène dépasse la normale, la plaque neurale largement étalée en surface est incapable de poursuivre sa morphogenèse quoique ses différenciations histologiques soient plus ou moins normales. Le caractère platyneurique du germe se décide durant le recul du nœud de Hensen et les premiers stades de la neurulation. Nos monstres platyneuriques âgés, dit Gallera, correspondent exactement aux anencéphales connus chez les Oiseaux et chez les Mammifères. Sur 36 embryons obtenus après exposition à l'action de l'atmosphère artificielle, des œufs, soit dès le début, soit à partir de la dixième heure, 8 seulement n'étaient pas platyneuriques.

Buchner, Rübsaamen, & Rothweiler (1951) ont obtenu des exencéphales sous l'influence du manque d'oxygène. Leurs expériences sont divisées en deux groupes. Dans le premier, la privation relative d'oxygène a été réalisée au cours des 24 premières heures de l'incubation; elle a donné des anencéphales.

Dans le second groupe, l'anoxie a été mise en œuvre après 36 ou 48 heures d'incubation, l'anencéphalie n'est plus apparue mais la microphtalmie et des malformations des membres et de la queue.

Rübsaamen (1954) a confirmé ces résultats et donné des figures montrant l'ouverture de l'encéphale. L'auteur signale 19 anencéphales sur 53 embryons.

Naujoks (1953), à la suite d'expériences semblables, se dit d'avis, comme Rübsaamen, que des malformations profondes n'atteignent l'ébauche cérébrale que quand le manque d'oxygène agit pendant les 24 premières heures. Ces auteurs sacrifient les embryons au septième jour, c'est-à-dire à un stade où la cléiencéphalie peut être facilement diagnostiquée. Ils ne signalent cependant aucun cas d'exencéphalie après action de l'anoxie à un stade dépassant 24 heures. Il apparaît ainsi que l'anoxie qui produit l'acléiencéphalie ne détermine pas la cléiencéphalie. Ce fait est d'accord avec nos résultats montrant que de ces deux malformations une seulement peut être produite par certaines substances chimiques.

Mushett (1953) ne signale pas, après privation d'oxygène, d'exencéphalie ni d'anencéphalie mais seulement des malformations oculaires.

En somme, les exencéphales obtenus par les auteurs paraissent être des acléiencéphales déterminés par l'anoxie.

(*b*) *L'exencéphalie chez les Mammifères*

Des malformations du crâne et de l'encéphale dans lesquelles la voûte du crâne était déficiente ont été signalées sous les dénominations d'anencéphalie, exencéphalie, craniochisis, encéphalocèle, pseudencéphalie par divers auteurs après action de différents agents tératogènes, mais les descriptions de la plupart des cas observés faisaient défaut ou ne permettaient pas de dire avec certitude s'il s'agissait de cléiencéphales ou d'acléiencéphales.

Snell & Picken (1935) signalent l'apparition de l'exencéphalie chez des embryons provenant de souris normales accouplées à des mâles soumis avant l'accouplement à l'action des rayons X.

Kaven (1938) a irradié des souris au huitième jour de la gestation et obtenu de nombreuses malformations parmi lesquelles il cite des hernies cérébrales et des dysencéphalies extracrâniennes rappelant d'après l'auteur la pseudencéphalie.

Snell (1941) a observé des exencéphales après irradiation de souris gestantes.

Warkany & Schraffenberger (1947) ont obtenu, après irradiation de 67 femelles de rat au treizième jour de la gestation, 19 embryons porteurs d'encéphalocèles, 40 d'un raccourcissement de la mâchoire supérieure et 40 de fissures palatines. Après irradiation aux quatorzième et quinzième jours, les auteurs ne signalent plus d'encéphalocèle.

Gillman, Gilbert, & Gillman (1948), après injection de trypan bleu à des femelles de rat gestantes, ont observé des malformations parmi lesquelles ils signalent: méningocèles, déficience des yeux, bec de lièvre, fissure palatine.

Ingalls, Curley, & Prindle (1950) ont étudié les effets de l'anoxie sur des souris gravides en les exposant à des atmosphères correspondant à celle qu'on rencontre à 25.000 pieds. Ils ont obtenu des malformations qu'ils dénomment anencéphalie tout en se demandant si le terme de craniochisis ne leur conviendrait pas mieux. Ces malformations seraient dues à un arrêt de développement au huitième jour de la gestation.

Russell (1950) décrit sous la dénomination de pseudencéphalie un cas apparu après irradiation (de 200 r) de souris au septième jour 1/2 de la gestation. Le cerveau élargi et saillant n'était pas recouvert par la peau. L'auteur signale d'autre part des embryons porteurs de 'cranial blisters' faisant saillie au travers de la voûte crânienne dans les régions frontale ou pariétale, unilatérales, bilatérales ou centrales; la peau les recouvre.

Werthemann & Reiniger (1950–1) ont observé sur des embryons de rat après anoxie des malformations des yeux et rarement des malformations de l'encéphale qui se limitaient au télencéphale.

Wilson & Karr (1951) ont irradié des femelles de rat au dixième jour de la gestation et citent parmi les malformations obtenues chez des embryons: 'méningocèles, encéphalocèles, craniochisis'.

Mme Lefebvre-Boisselot (1951) a étudié les effets de la déficience expérimentale en acide pentothénique sur la gestation de 132 femelles de rat de trois races différentes. Elle a obtenu des embryons anophtalmes, microphtalmes et exencéphales chez lesquels l'encéphale hernié au travers de la brèche due au non développement de la voûte crânienne 'est recouvert seulement et plus ou moins complètement par les méninges et la peau'. L'auteur signale 'des altérations de l'encéphale portant surtout sur le cerveau antérieur (télencéphale et diencéphale) et sur le mésencéphale. . . . L'organisation de l'encéphale peut être perturbée à des degrés divers de telle sorte que l'on passe ainsi de l'exencéphalie à ce qu'on peut appeler la pseudencéphalie.' 'Chez certains embryons, la structure de l'encéphale paraît se rapprocher de la structure habituelle. Dans certains cas, nous avons observé un encéphale à peu près normal si ce n'était la présence d'une hydrocéphalie asymétrique des ventricules latéraux amenant la paroi cérébrale à un aspect lamelleux. Cette hydrocéphalie paraissait consécutive à l'oblitération de l'aqueduc de Sylvius.'

Giroud (1952) décrit deux types parmi les exencéphales obtenus par Mme Lefebvre-Boisselot: 'Dans le premier, les structures encéphaliques peuvent être presque normalement développées, du moins on reconnaît les différenciations principales. Ce type peut être appelé exencéphalie. Dans l'autre type, les structures antérieures sont complètement bouleversées. On peut désigner ce type sous le nom de pseudencéphalie.'

Mme Lefebvre-Boisselot a signalé, et Giroud le rappelle, que 'dans certains cas, les cavités encéphaliques sont closes mais dans beaucoup d'autres elles sont ouvertes au moins en un point'. Nous avons donné plus haut les raisons pour lesquelles ces exencéphales doivent être divisés en cléiencéphales et acléiencéphales.

Les recherches de Hicks (1952) ont porté sur l'action des rayons X et de divers inhibiteurs ou antimétabolites sur le système nerveux central embryonnaire de rats et de souris. Les rayons X ont fait apparaître des malformations de l'encéphale que l'auteur dénomme encéphalocèle et anencéphalie partielle, accompagnées de malformations du crâne et de la face. La principale difformité résulte de la déficience de la partie supérieure du cerveau avec divers degrés de malformations de la tête. Les irradiations faites au neuvième et au dixième jours provoquent une déficience du cortex cérébral, une dilatation des ventricules (hydrocéphalie) et une déficience de la formation de l'hippocampe.

Ingalls, Curley, & Prindle (1952) complètent leur recherche de 1950 en donnant une étude anatomo-histologique pour démontrer que leurs cas d'anencéphalie sont identiques à ceux de Russell (1950) et Hicks (1952).

Hicks (1953) signale, après irradiation de souris gestantes au neuvième jour, de nombreuses malformations de la tête, de la face et du cerveau. L'auteur dénomme ces malformations anencéphalie, encéphalocèle, déficiences crânio-cérébrales et fait remarquer l'incorrection du terme anencéphalie.

Cohlan (1953) a fait ingurgiter de la vitamine A à 100 femelles de rat à l'aide d'un tube stomacal. 32.000 U.I. ont été introduites depuis le 2, 3, 4ème jour après coït jusqu'au 16ème. Les animaux ont été sacrifiés au 21ème ou 22ème jour ou plus près du terme. Sur 50 femelles, 44 produisirent des petits, 410 apparurent normaux (88 pour cent). Sur 100 femelles, 10 ont des petits à terme, au total 74; 36 d'entre eux ont des malformations du cerveau. La principale malformation apparente est la forte saillie de l'encéphale recouvert par une fine membrane. Les autres anomalies constatées sont la macroglossie, le bec de lièvre, la fissure palatine et des déficiences des yeux.

Katsunuma & Murakami (1954) ont injecté à des souris aux 6ème, 7ème, 8ème ou 9ème jour de la gestation 0,1 cm.^{3} de bleu trypan et au 8ème jour ces animaux divisés en 5 groupes ont été soumis à de basses pressions atmosphériques entre 250 et 400 mm. Hg. Les examens des embryons ont été faits au 13ème jour. Les auteurs ont observé comme malformations de la tête 'des pseudencéphalies (exencéphalies), craniochisis, encéphalocèles avec bec de lièvre et anophtalmie', mais ne donnent ni description ni statistique.

De l'ensemble de ces recherches expérimentales faites chez les Mammifères, il apparaît que l'exencéphalie peut être produite par des agents tératogènes divers et que ces exencéphales peuvent être des cléiencéphales ou des acléiencéphales. Mais l'insuffisance des descriptions rend rarement possible la distinction entre ces deux malformations et l'absence de statistique ne permet pas de comparaison pour les Mammifères avec celle que nous avons donnée pour les Oiseaux. Notre rapide exposé bibliographique nous paraît suffire pour montrer que ces deux malformations crânio-encéphaliques ne peuvent être différenciées par les appellations d'anencéphales et d'exencéphales à la suite de G. St. Hilaire ou par celles d'exencéphales et de pseudencéphales avec Mme Lefebvre-Boisselot et Giroud, mais par celles de cléiencéphales et d'acléiencéphales qui mettent seules en évidence le caractère fondamental qui les sépare.

SOMMAIRE

Des solutions de 4 substances chimiques ont été injectées en faible quantité sur la tête de jeunes embryons de poules Leghorn blanches. Deux variétés d'exencéphales ont été obtenues. Chez les uns, l'encéphale n'est pas fermé tandis

qu'il l'est chez les autres et entouré des méninges. Nous avons appelé les premiers acléiencéphales et les autres cléiencéphales et donné les raisons qui mettent en évidence la nécessité de remplacer par ces appellations les dénominations habituellement utilisées pour désigner les différentes variétés d'exencéphales. Elles qualifient deux malformations qui se réalisent à des stades différents et par des mécanismes différents. Diverses malformations de la face les accompagnent souvent, elles ont été décrites, classées et photographiées.

Un résumé a été donné des travaux des auteurs ayant obtenu l'exencéphalie à l'aide de différents agents tératogènes chez les Oiseaux et les Mammifères.

SUMMARY

Solutions of four chemical substances were injected in small amounts onto the heads of White Leghorn chick embryos. Two kinds of exencephaly were obtained. In one kind the brain was not closed off; in the other kind it was closed off and was surrounded by meninges. We have termed exencephalics of the first kind acleiencephalics, and of the second kind cleiencephalics, and have discussed the evidence which makes it necessary to replace the usual terms for the different varieties of exencephaly by these new terms. The new terms designate two malformations which appear at different stages and by different mechanisms. Various malformations of the face usually accompany them, and these are described, illustrated, and classified.

The work of previous authors on the production of exencephaly by various teratogenic agents in birds and mammals is summarized.

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EXPLICATION DES PLANCHES

PLANCHE 1

FIG. A. Cléiencéphale âgé de 17 jours, sans malformation de la face. Le cerveau antérieur et le cerveau moyen herniés sont nettement séparés l'un de l'autre. Ils sont revêtus par les méninges; une bande de peau sans duvet en continuité avec la peau normale entoure la hernie. Ricine 1:40.000, 1/24 mm.^{3} à 26 heures.

FIG. B. Cléiencéphale âgé de 20 jours, légère micrognathie supérieure et absence de la paupière supérieure. *A*: partie supérieure de l'occipital; *B*: extrémité inférieure du bec; *O*: orifice auditif. Colchicine 1:30.000, 1/8 mm.^{3} à 34 heures.

FIG. C. Acléiencéphale âgé de 7 jours. La partie antérieure des hémisphères cérébraux, surtout du gauche, est en continuité avec la peau qui les surplombe. Ricine 1:40.000, 1/24 mm.^{3} à 26 heures.

FIG. D. A gauche, acléiencéphale âgé de 8 jours. L'encéphale hernié fait une volumineuse saillie. Ricine 1:40.000, 1/24 mm.^{3} à 26 heures. A droite, acléiencéphalie spontanée chez un embryon de 9 jours. Vue postérieure montrant que tout l'encéphale est ouvert ainsi que la partie supérieure de la moelle. La ressemblance entre ces deux embryons et la rareté de nos cas expérimentaux avec ouverture de tout l'encéphale rend incertaine l'action de la ricine dans le cas de gauche.

FIG. E. Cléiencéphale âgé de 16 jours avec malformations de la face. Absence des yeux et du bourgeon frontal. Atrophie des bourgeons maxillaires supérieurs; langue courte; demi-bec inférieur normal. Trou auditif mis en évidence par arrachement du duvet. *A*, *B*, *O*: mêmes légendes que pour la figure B. Saponine S. 1:2.000, 1/8 mm.^{3} à 29 heures.

FIG. F. Cléiencéphale âgé de 21 jours. Absence des yeux. La hernie encéphalique est déviée vers la droite; elle repose sur le bourgeon frontal dont l'extrémité libre apparaît au-dessus de la langue. Les bourgeons maxillaires supérieurs atrophiés sont situés de chaque côté de la hernie et latéralement en arrière les deux orifices auditifs. Demi-bec inférieur normal. Trypaflavine 1:1.000, 1/8 mm.^{3} à 30 heures.

PLANCHE 2

FIG. G. Cléiencéphale âgé de 20 jours. Mêmes malformations de la face que dans la fig. F. La hernie encéphalique a été enlevée pour montrer la tige formée par le bourgeon frontal bien développé mais indépendant des bourgeons maxillaires supérieurs atrophiés. Ce bourgeon frontal a subi une torsion qui oriente à droite sa face inférieure. Trypaflavine 1:3.000, 1/12 mm.^{3} à 29 heures.

FIG. H. Cléiencéphale âgé de 21 jours. Au-dessus du maxillaire inférieur et de la langue normaux, on voit le demi-bec supérieur fortement dévié à gauche et recourbé. Il est formé par l'union du bourgeon frontal et du bourgeon maxillaire supérieur gauche. La hernie encéphalique est située entre lui et le bourgeon maxillaire droit atrophié, elle est recouverte par la dure-mère sauf dans une région arrondie. Trypaflavine 1:1.000, 1/8 mm.^{3} à 29 heures.

FIG. I. Cléiencéphale âgé de 19 jours, microphtalme bilatéral, micrognathe supérieur. La langue, cachée par le demi-bec supérieur, s'arrête au même niveau. La hernie encéphalique est formée par le cerveau antérieur non divisé en deux hémisphères et le cerveau moyen. Ricine 1:30.000, 1/24 mm.^{3} à 26 heures.

FIG. J. Cléio-acléiencéphale âgé de 12 jours. Microphtalme, micrognathe supérieur. Hernie du cerveau antérieur ouvert formant deux saillies latérales et du cerveau moyen revêtu des méninges. Conduit auditif largement ouvert. Trypaflavine 1:3.000, 1/8 mm.^{3} à 26 heures.

FIG. K. Cléiencéphale asymétrique âgé de 20 jours. Microphtalme droit, micrognathe supérieur, bec croisé. La hernie encéphalique est déviée à droite comme le bec. La ligne de séparation des deux hémisphères est oblique, sa partie antérieure déviée à droite. Trypaflavine 1:2.000, 1/8 mm.^{3} à 33 heures.

FIG. L. Acléiencéphale anophtalme âgé de 15 jours. Micrognathe supérieur, bec croisé dévié à gauche et dont la partie proximale fait défaut. Trypaflavine 1:1.000, 1/24 mm.^{3} à 26 heures.

PLANCHE 3

FIG. M. Cléiencéphale âgé de 20 jours. Les deux yeux font une forte saillie et ne sont pas recouverts des paupières. Micrognathe supérieur. Colchicine 1:30.000, 1/8 mm.^{3} à 34 heures.

FIG. N. Cléiencéphale âgé de 17 jours dont la face n'est représentée que par le demi-bec inférieur relevé vers la hernie encéphalique et dévié à droite. Grands orifices auditifs. Ricine 1:40.000, à 26 heures.

FIG. O. Cléiencéphale âgé de 11 jours. La hernie encéphalique touche le cœur. Pas de face sauf un petit diverticule placé sous la hernie et correspondant au bourgeon maxillaire inférieur. Colchicine 1:20.000, 1/24 mm.^{3} à 26 heures.

FIG. P. Acéphale, célosomien âgé de 18 jours. Absence des trous auditifs. La section passe en arrière, au ras des muscles de la nuque insérés sur l'occipital. Ricine 1:30.000, 1/12 mm.^{3} à 26 heures.

FIG. Q. Acléiencéphale âgé de 15 jours, anophtalme. Le demi-bec supérieur est divisé en deux parties rejetées latéralement et portant chacune une petite crête. Trypaflavine 1:1.000, 1/24 mm.^{3} à 26 heures.

FIG. R. Cléiencéphale, buphtalme du côté droit, âgé de 16 jours. Double crête formée de deux parties symétriques. Ricine 1:40.000, 1/24 mm.^{3} à 26 heures.

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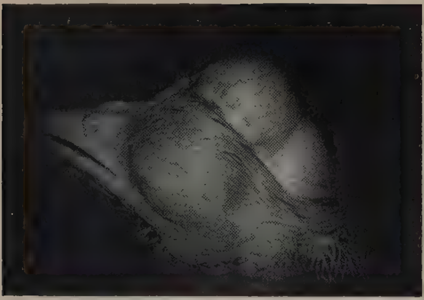

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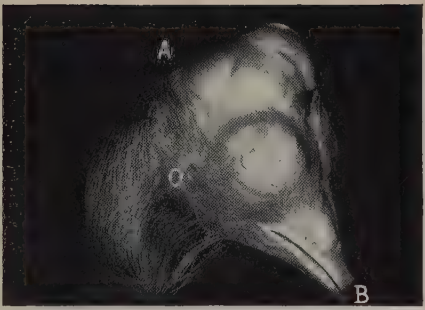

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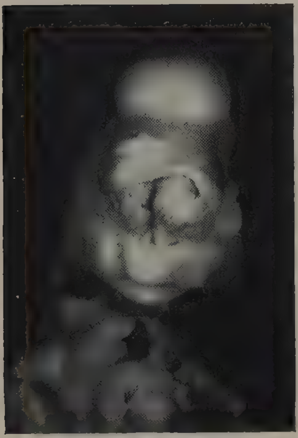

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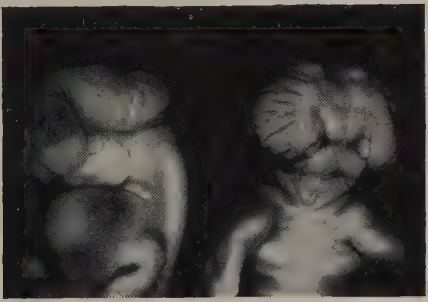

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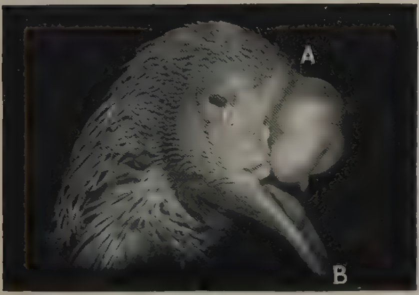

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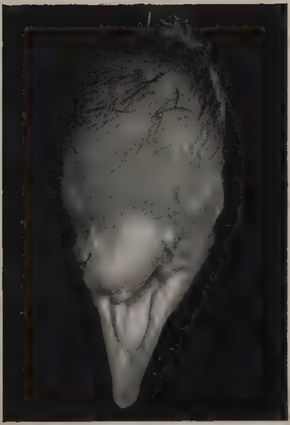

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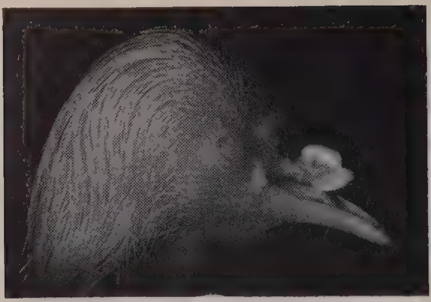
Planche 1



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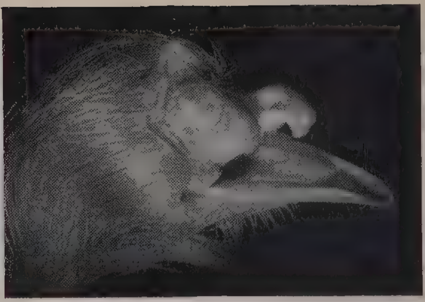

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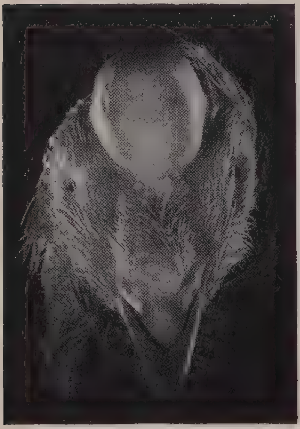

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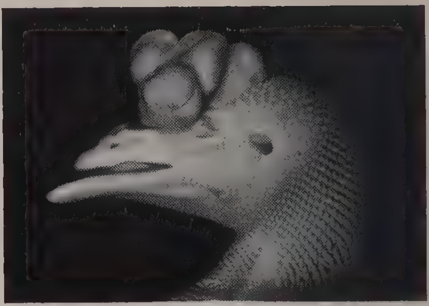

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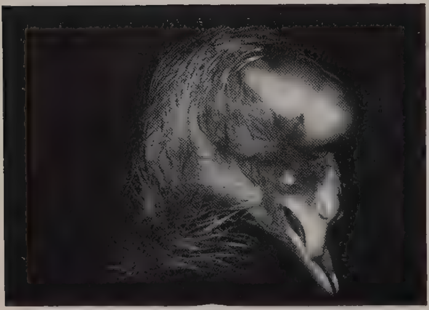

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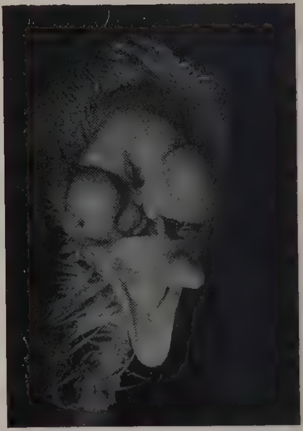

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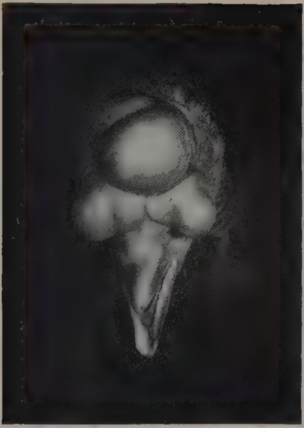
Planche 2



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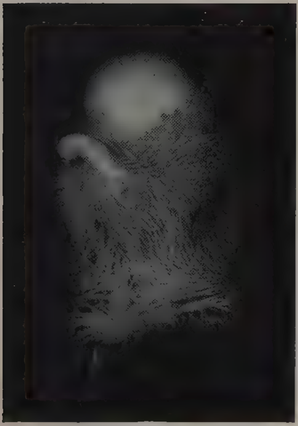

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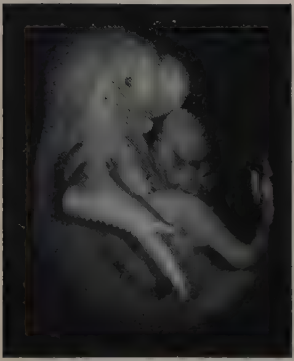

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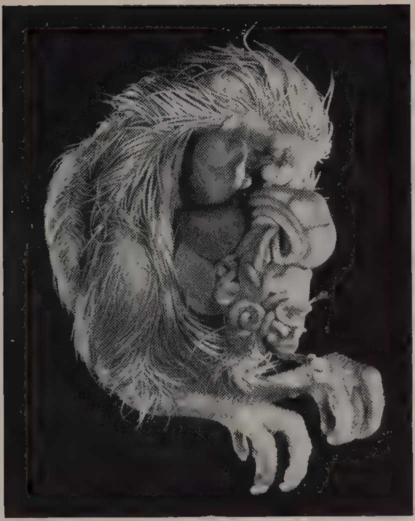

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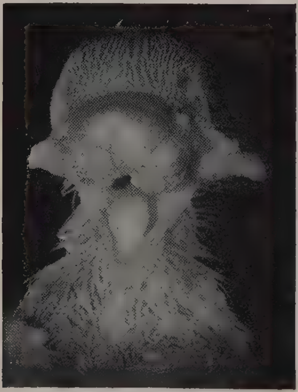

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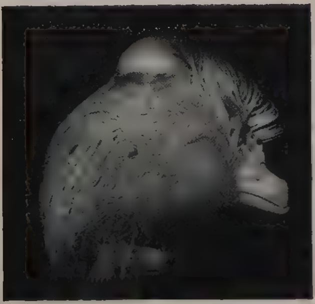

R

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Planche 3

The Reversibility of the Effect of Hypervitaminosis A on Embryonic Limb Bones Cultivated *in vitro*

by M. A. HERBERTSON[1]

From the Strangeways Research Laboratory, Cambridge

WITH TWO PLATES

INTRODUCTION

THE skeleton of young animals is profoundly affected by an abnormal amount of vitamin A in the body. In vitamin A deficiency changes in the functional activity of the osteoblasts and osteoclasts allow bone to be deposited in places where it would normally be removed, so producing excessive thickening of certain parts of the skeleton (Mellanby, 1938, 1939). Conversely, excessive vitamin A causes osteoporosis and spontaneous fractures, although the formation of new bone is not inhibited (Strauss, 1934; Wolbach & Bessey, 1942; Irving, 1949). Recent experiments have shown that the vitamin has a direct effect on skeletal tissues grown *in vitro*. Fell & Mellanby (1952) cultivated the long-bone rudiments of embryonic chicks and mice in medium containing vitamin A in concentrations similar to those found in the blood of animals suffering from hypervitaminosis A; in such explants the cartilage matrix lost its metachromasia and gradually disappeared (chicks, mice) while bone (mice) was rapidly resorbed.

In dogs fed on an A-deficient diet, the resulting skeletal changes could be at least partially reversed by restoring the vitamin to the food (Mellanby, 1947). The following investigation was undertaken to see whether the abnormalities produced in explanted bone-rudiments by the presence of excess vitamin A in the nutrient could be reversed in a similar way by transferring the bones to normal medium. It will be seen that under the conditions of the experiments most of the changes were not reversible, though they could be arrested.

MATERIAL AND METHODS

Explants

Chick embryos were removed from the egg after 6–7 days' incubation and the humerus, ulna, radius, femur, and tibia were explanted separately.

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The culture medium

Embryo extract was made from chicks of 13–16 days' incubation. The embryo was finely minced to form a pulp which was mixed with an equal volume of Tyrode's solution containing 1 per cent. glucose and centrifuged for 3 minutes. The extract was freshly prepared for each subculture. Another extract consisting of 1 part embryo : 2 parts of ordinary Tyrode's solution was also made and was used for washing the tissues.

An alcoholic solution of vitamin A was added to normal fowl plasma to give a concentration of about 2,000 i.u. or 4,000 i.u. of the vitamin per 100 ml. of plasma (+A plasma). The alcoholic solution of vitamin A gave a concentration of ethanol of 0·3 per cent. or less in the final plasma. Plasma from the same bird was used for the control cultures and ethanol was added to give the same concentration as in the +A plasma. The control plasma contained between 200 i.u. and 300 i.u. of vitamin A per 100 ml. The method of obtaining the plasma and of estimating its content of vitamin A has been described by Fell & Mellanby (1952).

The final culture medium consisted of a mixture of 3 parts plasma and 1 part embryo extract. Thus in the final medium the concentration of glucose in the embryo extract was lowered to 25 per cent. of its original value and that of vitamin A in the plasma to 75 per cent.; in the following account the values for vitamin A are those estimated in the original plasma.

The watch-glass method

The watch-glass method as described by Fell & Mellanby (1952) was used. Owing to the rapid rate of growth and consequent depletion of the medium it was necessary to subcultivate the chick explants four times each week. The tissues were explanted on 12 drops of medium, were transferred to 16 drops at the first subcultivation and continued at this level throughout the experiment; the ratio of 3 parts of plasma to 1 part of embryo extract was maintained throughout the culture period.

Measurements

Camera lucida drawings of the explants were made at the beginning of the experiments and at each subcultivation. Rough estimates of the size of the epiphyses of some bones were calculated from these drawings by using a planimeter to measure the surface area.

Histological technique

The explants were fixed in acetic Zenker's fluid for 1 hour, embedded in paraffin wax, and serially sectioned. Sections were stained with either Delafield's haematoxylin and chromotrop 2R, 0·5 per cent. toluidine blue in 5 per cent. alcohol, or by the azan method.

RESULTS

The object of these experiments (table) was to see if limb-bone rudiments cultivated for a time in hypervitaminotic medium could recover from the effects of the excess vitamin if transplanted to normal medium.

Although all the embryos used were of approximately the same age, their stage of development varied considerably. The structure of the rudiments when explanted is summarized in the table.

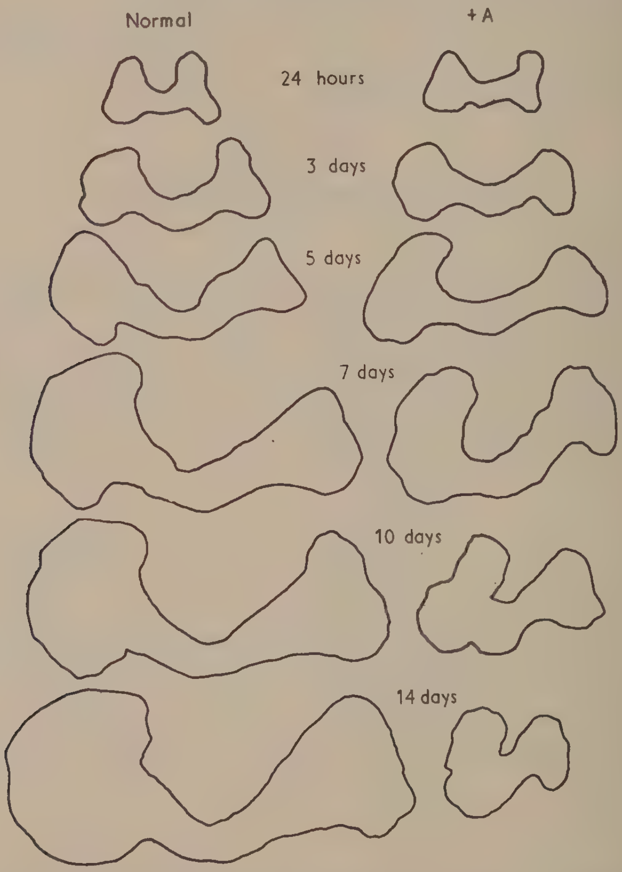
TABLE

Summary of Experiments 2–14

Experiment	*Vit. A i.u./100 ml. added to plasma*	*Embryo*	*No. of pairs of bone rudiments*	*Days in culture*		*Stage of development of rudiments at time of explantation*
				+A medium	*N medium*	
2	+A 2,000	1	4. Humerus, radius, femur, tibia	16 7	— 9	Chondroblastic hypertrophy visible in all explants.
3	+A 4,000	1	4. Humerus, ulna, femur, tibia	— 14	14 —	Chondroblastic hypertrophy visible only in femora and humeri.
	N 300	2	5. Humerus, ulna, radius, femur, tibia	5 5	2 9	
4	+A 4,000	1	4. Humerus, tibia, radius, femur	— 12	12 —	Chondroblastic hypertrophy in femora and humeri only.
	N 200	2	4. Humerus, ulna, radius, femur	4 4	3 8	
5	+A 2,000	1	4. Humerus, tibia, radius, femur	— 15	15 —	Chondroblastic hypertrophy distinct and periosteal bone visible in all explants.
	N 200	2	5. Humerus, ulna, radius, femur, tibia	4 4	2 11	
7	+A 2,000	1	5. Humerus, ulna, radius, femur, tibia	— 16	16 —	Chondroblastic hypertrophy visible in all explants.
	N 250	2	5. Humerus, ulna, radius, femur, tibia	6 6	2 10	Slightly more advanced than embryo 1.
14	+A 4,000	1	5. Humerus, ulna, radius, femur, tibia	— 18	18 —	Rudiments very small and soft. Chondroblastic hypertrophy visible only in femur.
	N 300	2	5. Humerus, ulna, radius, femur, tibia	9 9	2 9	

Experimental procedure

In Experiment 2 one embryo was used and one of each pair of rudiments was cultivated in +A medium for the whole of the culture period while the other was grown in similar medium for 7 days and then in normal medium for 9 days. In all the other experiments two embryos were used: from the first chick one of each pair of explants was cultivated in normal medium and the other in +A medium for the entire culture period; all the explants from the second embryo were cultivated in +A medium for 4–9 days and were then transferred to normal medium. After 2 or 3 days in normal medium one of each pair was fixed as a control and the other was maintained for the rest of the experiment. It was found that the vitamin A continued to exert an effect for 2–3 days after the bones were removed from the +A medium, probably owing to an accumulation of vitamin in the tissue; if, therefore, the control bones had been fixed when the others were

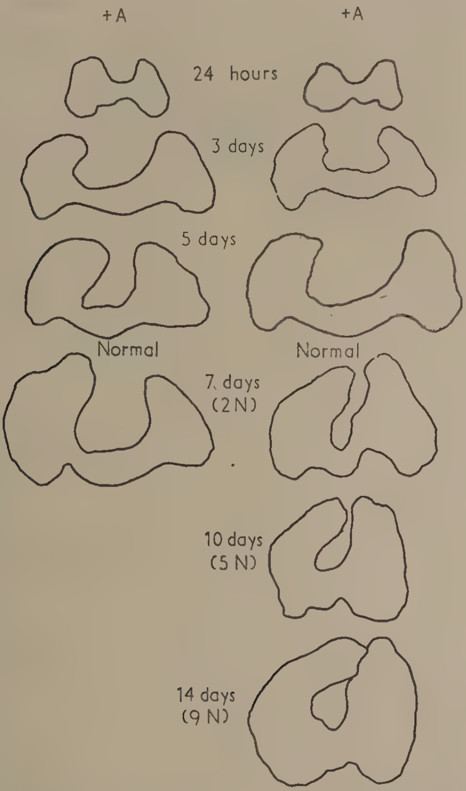


TEXT-FIG. 1. Camera lucida drawings of a pair of living humeri from a 7-day chick embryo (Exp. 3), one grown in normal and the other in +A medium (4,000 i.u./100 ml. plasma). The humerus in normal medium enlarged to more than three times its original length during 14 days' cultivation. That in +A medium grew normally at first but later shrank and became very distorted.

transferred to normal medium a true picture of the extent of the vitamin A effect would not have been obtained.

Behaviour of the rudiments in normal medium

The control rudiments cultivated in normal medium grew very well and usually increased to more than three times their original length (Text-fig. 1). The zones of hypertrophic cartilage cells, flattened proliferative cells, and epiphyseal cells differentiated in culture, and the layer of periosteal bone could be seen increasing in thickness and extending towards the ends of the diaphysis; there was no excavation of cartilage to form a marrow cavity. The soft tissue adhering to the bone grew rapidly.



TEXT-FIG. 2. Camera lucida drawings of a pair of living humeri from a 7-day chick embryo (Exp. 3). Both rudiments were grown in +A medium (4,000 i.u./100 ml. plasma) for 5 days and were then transferred to normal medium; one was fixed after 2 days and the other after 9 days in normal medium. Both the rudiments were affected by the excess vitamin and on transfer to normal medium there was no further growth of the shaft although the epiphyses continued to enlarge.

Sections of these bones fixed after 12–14 days *in vitro* showed that the shaft consisted of a middle region of large hypertrophied chondroblasts and the two zones of flattened cells beyond which the small-celled epiphyses could be distinguished (Plate 1, figs. A, E, H). Even after 15 days *in vitro* the cartilage cells were surprisingly healthy and there was little degeneration in either the shaft or epiphyses, but after 18 days some cells were necrotic. Mitoses were common in the epiphyses. The chondroblasts of the shaft were separated by thick partitions of matrix which was highly basophilic when stained with Delafield's haematoxylin and which stained metachromatically with toluidine blue. The layer of periosteal bone, which extended to the ends of the shaft, was usually very thick and occasionally some cartilage cells were enclosed in it. Most of the osteoblasts were healthy and they were usually arranged in fairly regular rows along the longitudinal axis of the rudiment.

Effect of hypervitaminosis

The explants grown in a concentration of vitamin A of about 2,000 i.u. per 100 ml. of plasma did not appear different from the controls until they had been in culture for 4 days, but those grown in a concentration of about 4,000 i.u. per 100 ml. were much more severely affected (Text-fig. 1). The cartilage of the hypervitaminotic explants differentiated into the usual three zones of cells and periosteal bone was formed quite as rapidly as in the controls but after about 4 days the growth rate declined and, as compared with the controls, the shafts became shorter and thinner; the epiphyses, although they continued to enlarge until about the seventh day, were also smaller than in the controls. Constrictions appeared at the ends of the shaft in the humerus and femur at about the sixth day in the lower concentration and at about the fourth day with the higher dose; by the end of the experiment usually all the bones were constricted and very distorted. After 7–8 days in culture the explants began to shrink although the surrounding soft tissue proliferated more extensively than in the normal medium.

Microscopical examination showed that after 4 or 5 days in +A medium the basophilia was usually lost from the periphery and ends of the shaft but remained in the centre of the diaphysis and in the epiphyses. After 7 days' cultivation, even in the lower concentration of the vitamin, the matrix of the shaft was usually completely colourless and the periphery of the epiphyses was also pale when stained with Delafield's haematoxylin. In the non-basophilic parts the matrix of the shaft was reduced, the cells were usually smaller than normal and there appeared to be more degenerate cells than in the areas which still stained with the haematoxylin. There were usually few degenerate cells in the epiphyses and mitotic figures were frequently observed.

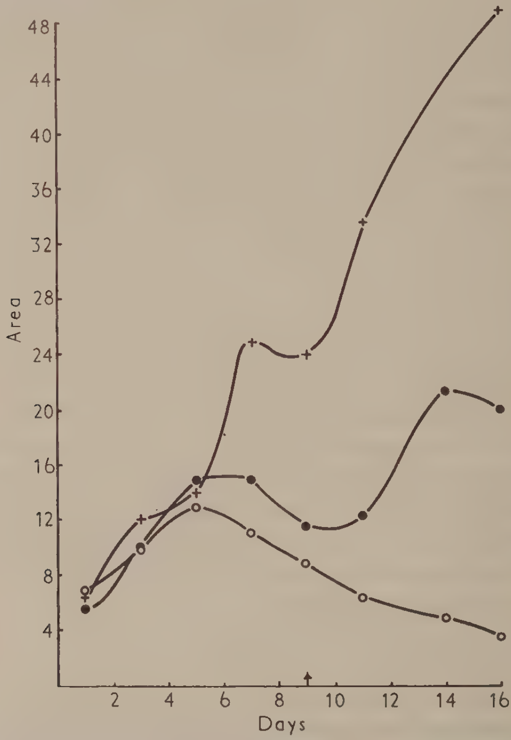
After 12–15 days in culture the histological differences between the treated and control explants were very great (Plate 1, cf. figs. A and E with B and F). In the +A medium most of the bones had become so distorted that it was difficult to distinguish the three zones of cells in the sections. There was usually as much periosteal bone as in the controls, but the osteoblasts, some of which were necrotic, were arranged in a less regular manner. The cartilage matrix showed most pronounced abnormalities. The amount of matrix between the chondroblasts in the shaft was reduced and had completely lost its basophilia and metachromasia and was colourless when stained with Delafield's haematoxylin or toluidine blue. The cells of the shaft were considerably smaller and less vacuolated than those of the controls and a higher proportion was necrotic. In places the matrix had disappeared completely and two or three chondroblasts were enclosed in one capsule; mitoses were sometimes observed in these cells (cf. Fell & Mellanby, 1952). After about 12 days in +A medium the cells of the shafts of some rudiments were completely necrotic and the matrix appeared to consist of a network of very loosely arranged fibres (Plate 1, figs. B and I). In other explants, however, the cells appeared quite healthy, mitotic figures were very frequently seen, and the intercellular substance was more compact (Plate 1, figs. F and G). The matrix of the epiphyses was less severely affected than that of the shaft, and basophilic and metachromatic areas were usually visible in the centre of the epiphyses although the periphery was pale. There were fewer degenerate cells than in the shaft but mitoses were seldom seen.

The extent of the changes produced by the excess vitamin A was directly related not only to the length of time spent in the +A medium but also to the stage of development of the rudiments when explanted, the younger primordia being more rapidly and more drastically changed than the older ones (Plate 1, cf. figs. B and I with figs. F and G). There were also differences in the effect on individual bones; the femur was always affected more than the other rudiments while the ulna and radius were the least changed.

The effect of transfer from +A to normal medium

The degree of recovery which the hypervitaminotic bones underwent when they were transferred to normal medium depended upon the severity of the damage they had sustained. In Experiment 5, for example, the rudiments were scarcely changed by their 4 days' contact with excess vitamin A and, when put into normal medium, they continued to grow and develop as well as the controls grown in normal medium throughout. This apparent lack of effect was partly due to the short time spent in +A medium but also to the facts that the lower concentration of the vitamin was used and that the rudiments were at an advanced stage of development when explanted (see table). In Experiment 4, however, which may be taken as a typical example, younger rudiments were explanted and after 4 days in the higher concentration of the vitamin they were severely affected (Plate 1, fig. C; Plate 2, fig. M).

When the rudiments were transferred to normal medium the changes produced by the hypervitaminosis were arrested (Plate 1, cf. figs. B and D; Text-fig. 2), but if the shaft had ceased to grow in the +A medium there was no further elongation after transfer although the width sometimes increased. The epiphyses were less drastically altered than the shaft and usually began to enlarge again in normal medium even though they had been shrinking previously (Text-fig. 2). Text-fig. 3 shows the relative sizes of the distal epiphyses of three comparable femora at intervals during Experiment 14 and the enlargement on transfer from +A to normal medium can be seen.



TEXT-FIG. 3. The sizes of the distal epiphyses of three comparable femora at intervals during Experiment 14. The epiphysis of the rudiment which was transferred to normal medium began to increase in size again although it had been shrinking while in the +A medium (4,000 i.u. vitamin A/100 ml. plasma).

↑ indicates transfer to normal medium.
+ = 16 days in normal medium.
○ = 16 days in +A medium.
● = 9 days in +A, 7 days in normal medium.

The transfer seemed to favour the formation of periosteal bone, and histological examination showed that in places a particularly thick layer was deposited which probably accounted for the increase in the width of the shaft mentioned above. Cartilage matrix which had disappeared from the diaphysis was not reformed nor was basophilia recovered where it had been lost (Plate 1, cf. figs. C and D). The diaphysial chondroblasts, however, largely recovered but although most of them looked healthy they were very different in appearance from the hypertrophic cells seen in the normal controls. The shaft as a whole resembled osteoid tissue rather than hypertrophic cartilage (Plate 2, figs. J and K), particularly in the region just beneath the layer of periosteal bone where the cells were very similar to the adjacent osteoblasts. In places the sheath of bone merged almost imperceptibly with the cartilage and the cells seemed to show a gradation from the young osteocytes in the osseous layer to the slightly hypertrophic cells in the interior of the shaft (Plate 2, cf. figs. J and L). Even the central chondroblasts, however, were smaller and less vacuolated than the hypertrophic cells of the controls (cf. Plate 2, fig. L and Plate 1, fig. H). In sections stained with Delafield's haematoxylin and chromotrop, the cartilage matrix which had lost its usual basophilia took up the pink of the chromotrop as did the periosteal bone. On the other hand, after Azan staining it acquired the pale blue coloration of normal cartilage and not the typical dark blue of osteoid tissue, but there was no sharp division of the colours where the character of the cells was changing as described above. The cartilage cells seemed to be more numerous than in the controls but this was probably due to crowding of the cells through loss of intercellular material, although mitoses were occasionally seen.

After the rudiments had been transferred to normal medium, many more mitotic cells could be seen in the epiphyses than in the explants grown either in normal or in high A medium throughout the experiment. The cells were similar in appearance to those of the normal controls and relatively few were necrotic but, as in the shaft, there was no restoration of lost matrix or of metachromasia.

DISCUSSION

These experiments show that excess vitamin A affects chick limb-bone rudiments cultivated *in vitro* in several ways. Differentiation of the cartilage cells continues but the growth of the rudiments is arrested and the cartilage matrix shrinks and loses its basophilia and metachromasia. Certain bones are affected more than others and the changes vary according to the stage at which the rudiments were explanted. These results confirm those of Fell & Mellanby (1952).

The changes produced by a few days' exposure to high vitamin A are only partly reversible by returning the explants to normal culture medium; the short treatment with the vitamin does not impair the viability of the chondroblasts and seems to increase their reproductive capacity (Lasnitzki, personal communication), but it inhibits their ability to form normal cartilage matrix even after the excess vitamin A has been withdrawn. Thus when the treated rudiments are returned to normal medium the epiphyses resume their growth, but their enlargement is mainly due to profuse mitosis; the matrix does not seem to increase in amount nor does it regain its former basophilia and metachromasia. In the shaft also there is no regeneration of matrix after the transfer, and although the chondroblasts recover their healthy appearance they do not regain their characteristic swollen, vacuolated structure; they resemble osteoblasts rather than chondroblasts and it is interesting that they sometimes undergo mitosis which is rarely, if ever, seen in normal, mature hypertrophic cells. Whether as a result of exposure to high vitamin A the chondroblasts have permanently lost their ability to form normal matrix or whether on prolonged cultivation in normal medium this function would eventually return, is not known.

SUMMARY

1. Experiments were made to study the reversibility of the effects of hypervitaminosis A on embryonic long-bones *in vitro*.

2. Limb-bone rudiments from 6- to 7-day chick embryos were cultivated by the watch-glass method, first in medium to which excess vitamin A had been added and then in normal medium. Suitable controls were also grown.

3. The excess vitamin arrested the growth of the explants and caused the cartilage matrix to shrink and lose its basophilia and metachromasia. The extent of the damage depended upon the length of time spent in +A medium, the concentration of the vitamin, the stage of development of the rudiment when explanted, and the particular rudiment treated.

4. The degree of recovery was related to the severity of the changes produced by the vitamin. In the shaft these changes were arrested but there was no restoration of lost matrix; the cells appeared healthy but resembled osteoblasts rather than normal hypertrophic chondroblasts. The epiphyses also partially recovered and began to enlarge again when transferred to normal medium; they had a high mitotic rate in comparison with the normal controls.

ACKNOWLEDGEMENTS

The author is greatly indebted to Dr. Honor B. Fell for suggesting this problem and for her help and advice, and to the late Sir Edward Mellanby for supplying the experimental plasma. She also wishes to thank Mr. V. C. Norfield, head assistant at the Strangeways Research Laboratory, for taking the photographs, Mr. L. J. King, technician at the Strangeways Research Laboratory, for technical assistance, and Mr. R. J. C. Stewart, chief technician of the Nutrition Building, National Institute for Medical Research, for the preparation of the plasma. Her thanks are also due to the Sir Halley Stewart Trust for a personal grant.

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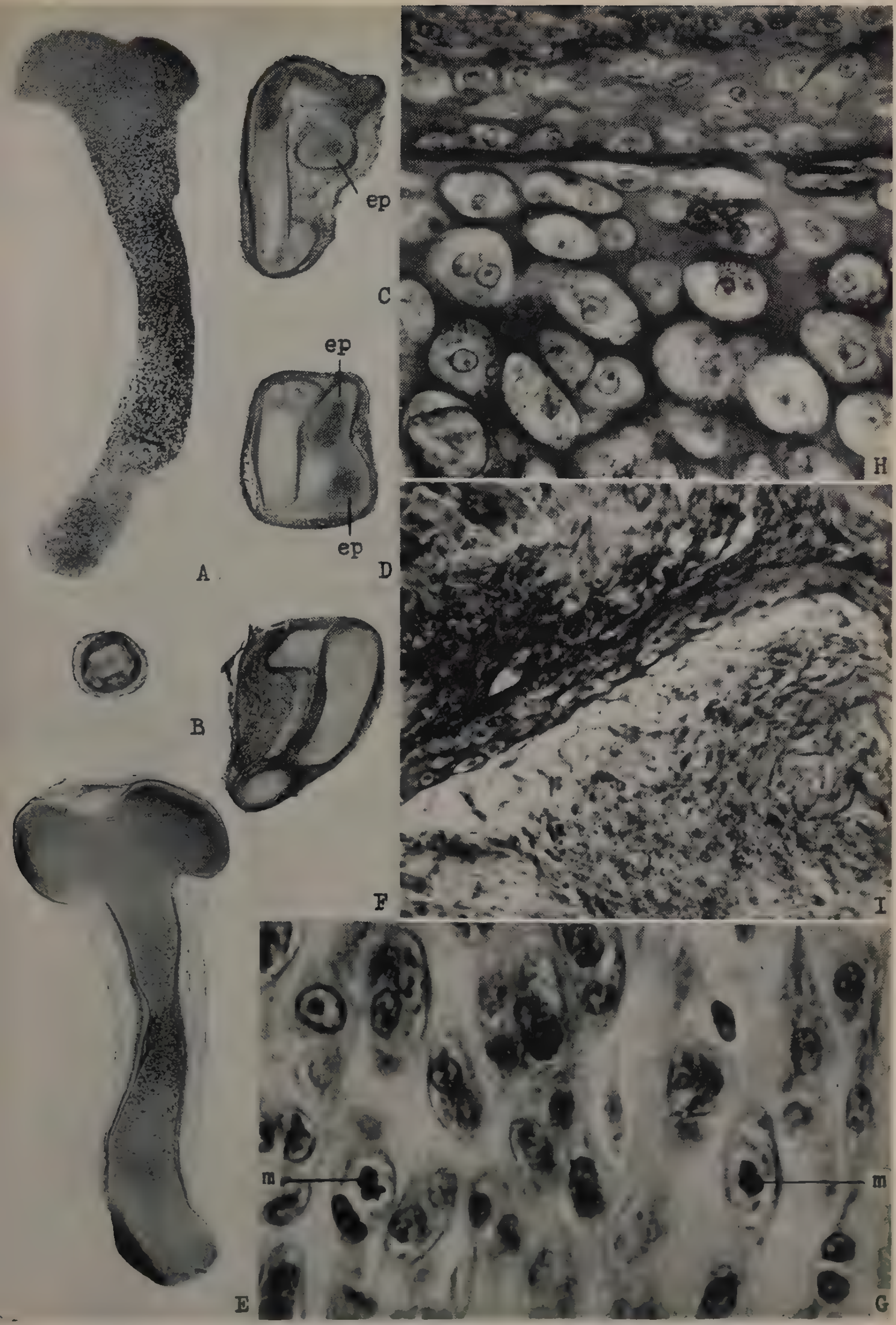
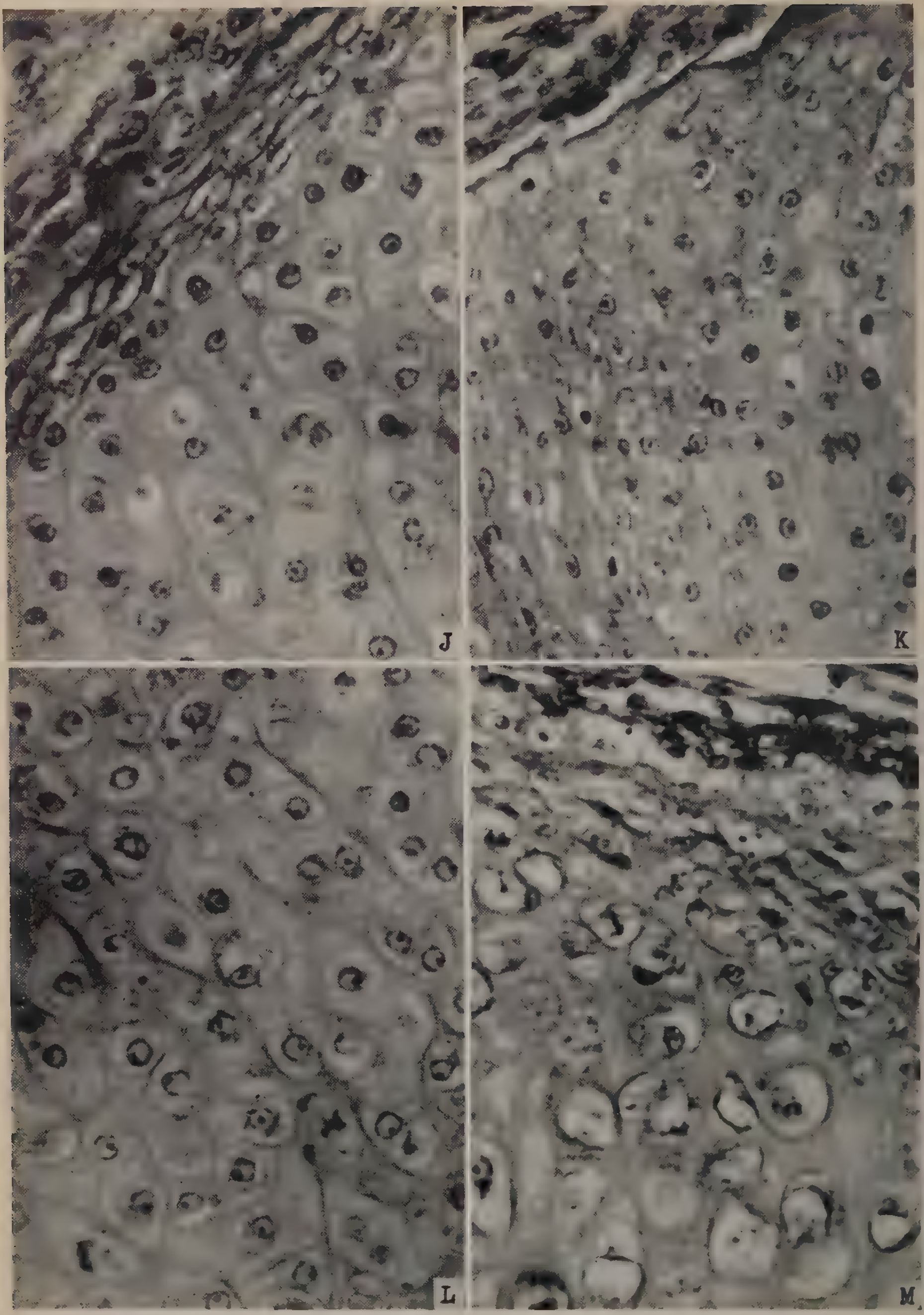


M. A. HERBERTSON

Plate 1



M. A. HERBERTSON

Plate 2

EXPLANATION OF PLATES

PLATE 1

FIG. A. Femur from Experiment 4 after 12 days' cultivation in normal medium. The differentiation of the chondroblasts can be seen and the cartilage matrix is strongly basophilic. ×17. (Delafield's haematoxylin and chromotrop.)

FIG. B. Femur from the opposite side of the same chick as shown in Fig. A after 12 days' cultivation in medium containing 4,000 i.u. vitamin A/100 ml. of plasma. The rudiment is very small, and the cartilage has almost disappeared (see Fig. I). ×17. (Delafield's haematoxylin and chromotrop.)

FIG. C. Femur from Experiment 4 after 4 days' cultivation in +A medium (4,000 i.u. vitamin A/100 ml. of plasma) and 3 days in normal medium. The bone is rather distorted and one epiphysis has almost disappeared. The basophilia has been lost from part of the shaft and from the outer region of the remaining epiphysis (*ep*) (see Fig. M). ×17. (Delafield's haematoxylin and chromotrop.)

FIG. D. Femur from the opposite side of the same chick as the explant shown in Fig. C after 4 days' cultivation in +A medium and 8 days in normal medium. The remaining epiphysis (*ep*) is much larger than the one seen in Fig. C, but there has been no recovery of basophilia in either the shaft or the epiphysis (see Figs. J, K). ×17. (Delafield's haematoxylin and chromotrop.)

FIG. E. Femur from Experiment 3 after 14 days' cultivation in normal medium. This shows the differentiation of the cartilage cells and the highly basophilic matrix (see Fig. H). ×17. (Delafield's haematoxylin and chromotrop.)

FIG. F. Femur from the opposite side of the same chick as shown in Fig. E after 14 days' cultivation in +A medium (4,000 i.u. vitamin A/100 ml. plasma). When explanted this rudiment was at a more advanced stage of development than that shown in Fig. B and was less changed by the vitamin A. The basophilia has been lost but the matrix is compact and most of the cells are healthy. ×17. (Delafield's haematoxylin and chromotrop.)

FIG. G. Part of the shaft of the femur shown in Fig. F. Groups of chondroblasts are enclosed in single capsules and two mitotic figures (*m*) can be seen. ×1,200. (Delafield's haematoxylin and chromotrop.)

FIG. H. Part of the diaphyseal region of the femur shown in Fig. E. The periosteum is at the top and beneath this is the sheath of periosteal bone with the osteocytes arranged in a fairly regular manner. The large hypertrophied chondroblasts are separated by thick bars of basophilic matrix. ×600. (Delafield's haematoxylin and chromotrop.)

FIG. I. Part of the femur shown in Fig. B. Many of the cells are necrotic and the matrix has disappeared almost entirely although a few pale fibres remain. The periosteal bone is less dense than in the normal control and many of the osteocytes are necrotic. ×170. (Delafield's haematoxylin and chromotrop.)

PLATE 2

FIG. J. Part of the shaft of the section shown in Fig. D. This shows the layer of periosteal bone which seems to merge with the cells of the shaft as the cells directly beneath the bone layer are very similar in appearance to osteoblasts. ×600. (Delafield's haematoxylin and chromotrop.)

FIG. K. Another region of the shaft of the femur shown in Fig. D. The chondroblasts are healthy but they are smaller and more compact than those of the normal controls. ×600. (Delafield's haematoxylin and chromotrop.)

FIG. L. The interior of the shaft of the femur shown in Fig. D. These cells also appear healthy and some are slightly hypertrophic but they are still smaller and less vacuolated than those seen in Fig. H. ×600. (Delafield's haematoxylin and chromotrop.)

FIG. M. Part of the shaft of the femur shown in Fig. C. The chondroblasts show a greater degree of hypertrophy than those seen in Fig. L. Some capsules contain more than one chondroblast and the matrix has lost its basophilia. ×600. (Delafield's haematoxylin and chromotrop.)

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Factors Affecting Vertebral Variation in Mice

2. Further Evidence on Intra-strain Variation

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INTRODUCTION

SINCE the enunciation by Johannsen in 1903 of the 'pure line' doctrine inbred strains have been used for various experimental purposes in the belief that they offer the highest attainable level of uniformity. This view is now known to be of limited validity. One reason is that 'pure lines' are not always as genetically pure as a simple Mendelian calculus would predict. Another is that for many characters inbred organisms are peculiarly sensitive during their development to environmental causes of variation (see the references cited by Grüneberg (1954), McLaren & Michie (1954*a*), and Biggers & Claringbold (1954); to these should be added Hagedoorn (1939), Mather (1946), Lerner (1954), and Kushner & Kameneva (1954)).

In this paper we consider the degree to which these two qualifications are exemplified by the variation of the number of lumbar vertebrae in mice.

TERMINOLOGY

As in the previous paper vertebral types will be denoted by the following symbols: 6/6 = six lumbar vertebrae on each side. 6/5 = six lumbar vertebrae on the left side and five on the right. 5/6 = five lumbar vertebrae on the left side and six on the right. 5/5 = five lumbar vertebrae on each side.

When we wish to characterize a particular strain or substrain as having predominantly 5 or predominantly 6 lumbar vertebrae, we shall add the number 5 or 6 in parentheses.

GENETIC DIFFERENCES BETWEEN COLONIES OF C3H(5) MICE

In our survey of the C3H strain (McLaren & Michie, 1954*b*) we showed that within the part of the strain which has predominantly 5 lumbar vertebrae (substrains C3H/St and C3H/Bi) significant differences exist in the actual percentages shown by different colonies. Our data were not adequate to exclude the possibility that the differences were due entirely to differences in the environmental conditions in which different laboratories maintained their colonies. This

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possibility was supported by the fact that vertebral type is affected by some factor of the mother's physiology which fluctuates during her lifetime (see McLaren & Michie, 1954*b*, and also below) and by Searle's (1954*a*) observation that some skeletal characters in the mouse are affected by changes in the mother's diet.

TABLE 1

The substrain, origin, and vertebral types of three C3H(5) *colonies and of our* C57BL *colony. The information on the* C3H *substrains includes that given by McLaren & Michie (1954*b*). The* C57BL *data are new. For each sample the top rows of figures give the actual numbers of mice and the bottom row gives percentages*

		Vertebral types				
Substrain	*Origin*	5/5	6/5	5/6	6/6	*Total*
C3H/St	Wilson in 1952 ♀♀	22	0	0	0	22
	♂♂	38	0	0	0	38
	not sexed	29	1	0	0	30
		89	1	0	0	90
		98·9	1·1	0·0	0·0	100·0
C3H/Bi	Green in 1952 ♀♀	15	0	0	1	16
	♂♂	26	1	0	0	27
		41	1	0	1	43
		95·3	2·3	0·0	2·3	99·9
C3H/Bi	Craigie in 1953 ♀♀	17	7	2	4	30
	♂♂	42	2	1	0	45
		59	9	3	4	75
		78·7	12·0	4·0	5·3	100·0
C57BL/How	Howard in 1950 ♀♀	0	0	0	112	112
	♂♂	1	0	1	74	76
		1	0	1	186	188
		0·5	0·0	0·5	98·9	99·9

In order to discover whether, apart from such causes of variation, genetic differences have arisen between colonies, we crossed males from each of the C3H(5) colonies listed in Table 1 to females of our C57BL colony. The C3H/St and C3H/Bi substrains have been separated since 1931. Green's and Craigie's colonies of the C3H/Bi substrain have been separated since 1938 (see McLaren & Michie, 1954*b* for full genealogy).

The three sets of matings were set up so as to run concurrently, and their F_1 offspring were classified for vertebral type. The results are shown in Table 2. There are marked differences between the three sets of offspring, as indicated by the differences between the percentages given in the last column of the table. These differences are statistically highly significant.

The appropriate statistical test is a $\chi^2_{(2)}$ corresponding to heterogeneity between the three mating types. The calculated value is shown in Table 3, in which a full χ^2 analysis of the data is set out. We cannot assess the significance of this value of $\chi^2_{(2)}$ directly from a table of χ^2, because there is significant heterogeneity between litters *within* mating types. This is shown in Table 3 by the large value of the corresponding $\chi^2_{(85)}$. We must therefore divide each of these χ^2 values by the number of its degrees of freedom, and assess the ratio of the two mean squares so obtained from a table of the variance ratio (Fisher & Yates, 1938–53). This procedure yields a value which is significant at the 1 per cent. probability level, as shown in the table. General principles for partitioning and assessing χ^2 values in cases of this sort have been described by Howard, McLaren, Michie, & Sander (1955).

TABLE 2

The results of crossing males of three different C3H(5) *colonies to* C57BL *females*

Origin of males		*Vertebral types of F_1 offspring*				*Total*	*6/6 as per cent. of total*
		5/5	6/5	5/6	6/6		
C3H/St from Wilson	♀♀	25	9	1	22	57	
	♂♂	16	9	2	17	44	
		41	18	3	39	101	38·6
C3H/Bi from Green	♀♀	19	11	3	56	89	
	♂♂	30	14	3	46	93	
		49	25	6	102	182	56·0
C3H/Bi from Craigie	♀♀	6	4	3	48	61	
	♂♂	16	8	2	34	60	
		22	12	5	82	121	67·8

TABLE 3

χ^2 analysis of the data summarized in Table 2

	χ^2	*DF*	*MS*	
Between litters within matings	93·7452	55	1·7045	Mean square ratio = 1·240 $P > 0·2$
Between matings within mating types	63·4259	30	2·1142	
Between litters within mating types	157·1711	85	1·8491	Mean square ratio = 5·172 $P < 0·01$
Between mating types	19·1260	2	9·5630	
Total heterogeneity between litters	176·2971	87		
Overall deviation from 56·4%*	0·2414	1		
TOTAL	176·5385	88		

* This value was taken for the proportion of 6/6 in the three sets of data combined. It is slightlyin error owing to the inclusion of some subsequent litters. The discrepancy is of negligible effect on the statistical analysis.

Before concluding that there is significant genetic variation between the three C3H(5) colonies from which the tested males were drawn, we must be sure that the differences do not arise from heterogeneity between the three samples of C57BL females to which they were crossed. The C57BL females were all descended from four original matings made in 1950. When the F_1 offspring were classified into four groups according to maternal origin, no consistent differences in vertebral type were apparent. Consequently we judge the differences between the three sets of data shown in Table 2 to be evidence that the three C3H(5) colonies from which the males were drawn have diverged genetically during the period of their spatial separation.

This result is thus fully in harmony with the generalization made by Grüneberg (1954) in summarizing work on skeletal variation on mice: 'There is evidence that pure lines of mice are much less homogeneous genetically than is widely believed.' The point is of considerable importance for experimental biologists who use inbred strains in their work. Reliance is customarily placed upon the constancy over long periods of time of the biological properties of inbred material, and on the effective genetic identity of recently separated colonies or substrains of the same strain. This view should be qualified by the realization that sometimes quite rapid evolutionary change may occur even in inbred strains. Billingham, Brent, Medawar, & Sparrow (1954) have been able to detect antigenic differences between sublines of an inbred strain after as few as four generations have elapsed since their separation.

Two mechanisms may be involved: (1) heterozygosity may persist in spite of inbreeding owing to inferior viability and fecundity of homozygous genotypes; (2) current ideas of the frequency of mutation are based on the study of mutations of major effect. The frequency of the mutations of small effect which underlie multifactorial variation may be much higher. The smaller the individual effects of mutation the higher is the frequency which can be tolerated without detriment to the evolutionary fitness of the population.

Other causes of variation in the above crosses

In an earlier paper (McLaren & Michie, 1954*b*) we described a phenomenon in matings within the C3H/He substrain which we termed 'clumping'—a tendency for members of the same litter to resemble one another in vertebral type. Our data suggested a true heterogeneity between litters of the same mating rather than a secondary effect of heterogeneity between different matings. The effect was not attributable to systematic changes in maternal physiology associated with maternal age, birth order, or litter size. We inferred that vertebral type was sensitive to a fluctuating variable of the mother's physiology as in the case of a tooth abnormality described by Grüneberg (1951).

The new data on interstrain F_1 hybrids summarized in Table 2 decisively confirm the postulated effect. This is shown by the χ^2 corresponding to heterogeneity between litters within matings in Table 3. It has the value of 93·7452 for 55 degrees of freedom, for which P is less than 0·001. There is no significant evidence of heterogeneity between matings over and above this heterogeneity between litters. This is shown by the comparison between the two corresponding mean squares, which yields a variance ratio of 1·240 ($P > 0{\cdot}2$). Nor is any significant part of the heterogeneity attributable to systematic changes associated with maternal age, birth order, or litter size. The corresponding χ^2 values are respectively 0·789, 1·540, and 1·614, each for one degree of freedom.

These data on inter-strain crosses therefore support the suggestion made on the basis of scantier data from intra-strain matings that vertebral type is in part determined by factors acting on prenatal development which fluctuate during the mother's lifetime. They also show that this effect is exerted by C57BL as well as by C3H females, since the new data relate to the offspring of C57BL mothers. In addition these data agree with the intra-strain results in that the variation of the left and right sides is positively correlated, the 6/5 type predominates over the 5/6 type, and males tend on average to have fewer lumbar vertebrae than females. All the effects discussed in this section have also been found by Searle (1954*b*) in intra-strain matings of C57BL/Gr mice.

If we wished to be entirely rigorous in assessing the differences between the bodies of data shown in Table 2 we should perhaps attempt to allow for the sex difference, instead of pooling data from the two sexes. But the observed sex differences are not large, and inspection of the actual sex ratios shows that the conclusions cannot have been affected by ignoring the sexes of the mice when comparing bodies of data.

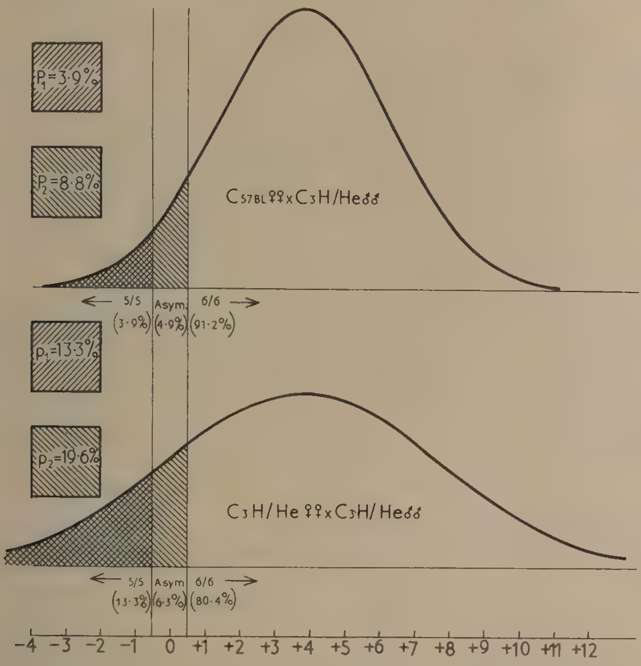
THE PHENOTYPIC VARIABILITY OF INBRED AS COMPARED WITH HYBRID MICE

We have mentioned above that for many characters in several species of plants and animals it has been found that the genetic homozygosity brought about by inbreeding is associated with increased vulnerability to non-genetic causes of variation. It is not yet known how to predict from the nature of the character under study whether or not inbreeding will result in developmental instability of this type. In this section we inquire whether vertebral type is such a character.

Method of analysis

For this purpose three grades of expression of the character can usefully be distinguished: 5/5, asymmetrical and 6/6. At first sight it might seem impossible to measure the variability of a sample of animals for a character which does not vary on a continuous scale. But since vertebral type is determined by the joint action of a large number of genetic and environmental factors, it should belong to the class of characters which Grüneberg (1952 *a*, *b*) has termed 'quasi-continuous'. That is to say, we regard the discontinuous categories into which the character is classified as marking threshold values of some underlying continuous distribution.

We thus assume that in mice there is a continuous gradation of the tendency towards higher or lower numbers of lumbar vertebrae which could in principle be precisely defined for each mouse, but which in practice can only be measured as lying to one side or the other of the threshold separating the 5/5 from the asymmetrical, or the asymmetrical from the 6/6, expression of the character. If the multiple factors influencing vertebral type act approximately additively, we can assume the mice to be normally distributed on the underlying continuous scale. It is then easy to estimate the mean and standard deviation on this scale of any sample of mice in which all three vertebral types are represented. The principles of estimation have been described by Wright (1934) and applied to vertebral variation by Green (1951).



TEXT-FIG. 1. The distribution of the three vertebral types—5/5, asymmetrical and 6/6—in samples of C3H inbred mice (lower curve) and C57BL♀ ×C3H♂ F_1 mice (upper curve). Normal frequency curves have been drawn to fit the observed percentages of vertebral types, which are represented by shaded areas of the two curves. Note the greater spread of the lower curve.

p_1=percentage of 5/5, p_2=percentage of non-6/6 (5/5+asymmetrical). For fuller explanation see text.

We shall arbitrarily fix the scale so that a population containing equal numbers of 5/5 and 6/6 types has its mean at zero, and we shall take the distance between the two thresholds as unity. In Text-fig. 1 a sample of F_1 inter-strain hybrids is depicted graphically on this scale, together with a sample of one of the parental strains. It will be noticed that, for a given mean, the spread of the distribution (i.e. the variability of the mice in the sample) is inversely related to the frequency of asymmetry. If Y_1 and Y_2 are the probits (Fisher & Yates, 1938–53, Table IX) of the proportions p_1 and p_2 defined in the text-figure, the mean can be estimated as

$$m = \frac{5-Y_1}{Y_2-Y_1} - \frac{1}{2}$$

and the standard deviation as

$$s = \frac{1}{Y_2-Y_1}.$$

In what follows it has been more convenient to use estimates of $1/s$, given by the probit difference $Y_2 - Y_1$.

The data

In Table 4 this quantity, $Y_2 - Y_1$, has been tabulated for those colonies of the C3H and C57BL inbred strains and their F_1 and F_2 hybrids on which information on vertebral type has been published. Only samples in which both the 5/5 and 6/6 types are represented have been included. $1/s$ has been estimated separately for each of the three groups—inbred, F_1 and F_2—from the weighted mean of the samples within the group.

Method of weighting and combining the estimates

The estimate of $1/s$ yielded by a sample of n mice has a sampling variance

$$1/n\left(\frac{p_1q_1}{z_1^2} - \frac{2p_1q_2}{z_1z_2} + \frac{p_2q_2}{z_2^2}\right),$$

where z_1, z_2 are the ordinates of the normal distribution at Y_1, Y_2 (Fisher & Yates, 1938–53, Table II). This expression is taken, with a notational change, from Haldane (1955), and is well approximated by the more manageable expression Q/nZs where $P = 1 - Q =$ the proportion of asymmetrical types, and Z is the mean of z_1 and z_2. We have taken the reciprocal of this expression, i.e. $nZs/Q = I$, as the amount of information contributed by each sample, using a joint working estimate of s for all samples within each group, and adjusting the values of Q accordingly.

Discussion

It appears from Table 4 that the C3H and C57BL inbred strains are indeed more variable than their F_1 hybrids. The joint estimate of the standard deviation for inbreds is greater than that for F_1 hybrids, and the difference is statistically highly significant ($P < 0{\cdot}005$). In terms of the variance, s^2, the inbreds are almost twice as variable as the F_1 hybrids.

There are two unsatisfactory features of the data. (1) Most of the information on the level of inbred variability comes from one of the two parental strains.

TABLE 4

Estimates of variability in the C3H *and* C57BL *inbred strains and their* F_1 *and* F_2 *hybrids. For explanation see text*

Strain and substrain	*Origin*	*No. of mice*	Y_2-Y_1	*Information* = I
C3H/St[2]	Strong in 1937	200	0·575	28·6
C3H/Bi[2]	Bittner in 1939 (= Bittner's Z)	766	0·445	91·7
C3H/He[2]	Law in 1948	176	0·295	26·3
C3H/HeRl[2]	Russell in 1950 (Oak Ridge)	72	0·469	11·5
C3H/Bi[2]	Bittner in 1950 (= Bittner's Z)	144	0·974	27·9
C3Hf/Bi[2]	Bittner in 1950 (= Bittner's Zb)	75	0·068	38·8
C3H/St[3]	Strong in 1953	38	0·379	37·5
C3H/Bi[5]	Green in 1952	43	0·302	8·6
C3H/Bi[3]	Dmochowski in 1953	26	0·517	21·6
C3H/Bi[3]	Foulds in 1951	26	0·407	13·3
C3H/Bi[4,5]	Craigie in 1953	75	0·820	40·2
C3H/He[3]	Hewitt in 1951	29	0·000	23·2
C3H/He[4]	Howard in 1950	158	0·256	107·1
C57BL[1]		395	0·388	53·7
C57BL/How[4, 5]	Howard in 1950	188	0·250	9·7
		2411		539·7

For all inbreds combined $1/s$ is $0{\cdot}403 \pm 0{\cdot}043$, $s = 2{\cdot}484$, $s^2 = 6{\cdot}170$.

F_1 *hybrids*	*No. of mice*	Y_2-Y_1	*Information* = I
C3H♀♀×C57BL♂♂[1]	349	0·424	292·2
C57BL♀♀×C3H♂♂[1]	641	0·653	551·1
C57BL/How♀♀×C3H/St♂♂[6]	101	0·528	92·3
C57BL/How♀♀×C3H/Bi♂♂[6]	182	0·465	153·3
C57BL/How♀♀×C3H/Bi♂♂[4,6]	121	0·446	84·2
C3H/Bi♀♀×C57BL/How♂♂[4]	51	0·523	33·5
C57BL/How♀♀×C3H/He♂♂[4]	102	0·409	24·4
C3H/He♀♀×C57BL/How♂♂[4]	65	0·786	41·2
	1612		1272·2

For all F_1 hybrids combined $1/s$ is $0{\cdot}551 \pm 0{\cdot}028$, $s = 1{\cdot}814$, $s^2 = 3{\cdot}291$.

F_2 *hybrids*	*No. of mice*	Y_2-Y_1	*Information* = I
F_2(C3H×C57BL)[1]	1278	0·305	1871·1

For F_2 $1/s$ is $0{\cdot}305 \pm 0{\cdot}023$, $s = 3{\cdot}279$, $s^2 = 10{\cdot}752$.

For each group $1/s$ is estimated as $\dfrac{\sum I(Y_2-Y_1)}{\sum I} \pm \dfrac{1}{\sqrt{(\sum I)}}$.

Sources of data:

1. Green & Russell (1951), 2. Green (1953), 3. McLaren & Michie (1954*b*), 4. McLaren & Michie (1956), 5. Table 1 above, 6. Table 2 above.

Information on the other, C57BL, is scanty. (2) The effects of small errors in the assumptions on which the continuous scale is constructed will be greater for samples which contain a very small proportion of either the 5/5 or the 6/6 type. Very few of the hybrid, but about half of the inbred, samples fall into this category.

If, however, the difference between the inbred and F_1 distributions is taken at its face value, it must be related almost entirely to a difference in the reactivity to *non-genetic* causes of variation of homozygous as compared with heterozygous genotypes. This is because both inbred and F_1 populations share approximately the same, very low, level of genetic variation. On the other hand, in the F_2 generation we may expect the total variability to exceed the F_1 level owing to the release by segregation of *genetic* variability. This expectation is fulfilled by the data of Table 4, from which it appears that the F_2 mice are about $3\frac{1}{2}$ times as variable as the F_1 hybrids and about 1·8 times as variable as the inbreds. Some support is thereby given to the assumptions on which the method of analysis rests.

A connexion between hybrid uniformity and heterosis or hybrid vigour has sometimes been suggested. Robertson & Reeve (1952) have put the matter as follows:

> '. . . the more heterozygous individuals will carry a greater diversity of alleles, and these are likely to endow them with a greater biochemical versatility in development. This will lead to heterosis, because of the more efficient use of the materials available in the environment, and also to a reduced sensitivity to environmental variations, since there will be more ways of overcoming the obstacles which such variations put in the way of normal development.'

From this it might wrongly be inferred that all characters which show hybrid uniformity will also manifest heterosis—in the sense of having a more extreme mean value in the F_1 than that of either parental population. This inference is valid only for characters with an adaptive optimum of which both parental types fall short, e.g. fecundity, viability: here greater developmental efficiency must necessarily be manifested in heterosis in the sense defined above. The inference does not hold for other characters, which may reasonably be expected to include vertebral type; and indeed, with the single exception of the C3H/He♀ × C57BL/How♂ cross, the vertebral types of the F_1 hybrids fall within the range separating the parental means.

SUMMARY

1. As a test for genetic differences within that part of the C3H inbred strain of mice which has predominantly 5 lumbar vertebrae, males from each of three C3H(5) colonies were crossed to C57BL females.

2. The F_1 offspring differed in vertebral type according to paternal origin, showing that genetic divergences between colonies and substrains have occurred within that section of the C3H strain which has predominantly 5 lumbar vertebrae.

3. These matings also gave further evidence, in the form of significant heterogeneity between litters, that vertebral type is in part determined by a fluctuating variable of the mother's physiological state.

4. We have summarized published data on the variation of the number of lumbar vertebrae in mice in the C3H and C57BL inbred strains and their F_1 and F_2 hybrids. On certain assumptions we find that the inbreds are about twice as variable as the F_1 hybrids.

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The Development of the Ultimobranchial Body in *Xenopus laevis* Daudin and its Relation to the Thyroid Gland and Epithelial Bodies

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WITH TWO PLATES

INTRODUCTION

THE ultimobranchial body is a derivate of the pharyngeal cavity which has been found in all vertebrates studied at least at some stage of development. Its origin, development, and structure has been studied in many species but nevertheless nothing certain is known about its significance and activity. Earlier authors have used several terms for this organ, e.g. accessory thyroid gland, lateral thyroid, corpus Y, suprapericardial body, telobranchial body, and postbranchial body. The term mostly used at present, ultimobranchial body, was suggested by Greil (1904).

It is not possible to give a consistent picture of the formation of the ultimobranchial body, since it is evidently different in different vertebrates. In fishes it is formed from the sixth rudimentary visceral pouch (van Bemmelen, 1889); in amphibians Maurer (1888) regards it as being formed from the ventral wall of the pharyngeal cavity, while Greil (1904) derives it from the sixth visceral pouch. In reptiles it develops as a common bud together with the fifth and sixth visceral pouch (Johnson, 1922). In birds, according to Rabl (1907), it develops from the fifth and sixth visceral pouches, or else from the sixth pouch only (Sicher, 1921; Dudley, 1942). Opinion in regard to the mammals varies. Tandler (1909) derives it from the fifth visceral pouch, Lewis (1906) and Klapper (1946), in contrast, from the fourth visceral pouch. Kingsbury (1914, 1915) considers that the ultimobranchial body does not represent a visceral pouch at all.

The later development of the ultimobranchial body in the lower vertebrates is in general similar. Laterally to the larynx it forms a pair of uni- or multilobar organs, most nearly resembling the thyroid gland (Watzka, 1933, &c.). The size, structure, and localization of the organ vary considerably and in Urodeles, for

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instances, only the left body is developed (Wilder, 1929). The later development of the ultimobranchial body in mammals is incompletely known. It is evident that it does not form an independent, persistent organ. Most authors agree that during development it fuses with the lateral thyroid lobes, forming there rudimentary cysts (Rabl, 1931; Politzer & Hann, 1935; van Dyke, 1944, 1949, 1955) or ultimately disappears altogether (Grosser, 1910; Kingsbury, 1914, 1935, 1939; Klapper, 1946). It is an opinion fairly generally held that the ultimobranchial body forms active thyroid tissue (Badertscher, 1918; Rogers, 1929; Weller, 1933; Massart, 1940) and that the thyroid gland in mammals is thus formed from three anlagen, one median and two lateral ones. This theory, however, is unproved.

MATERIAL AND METHODS

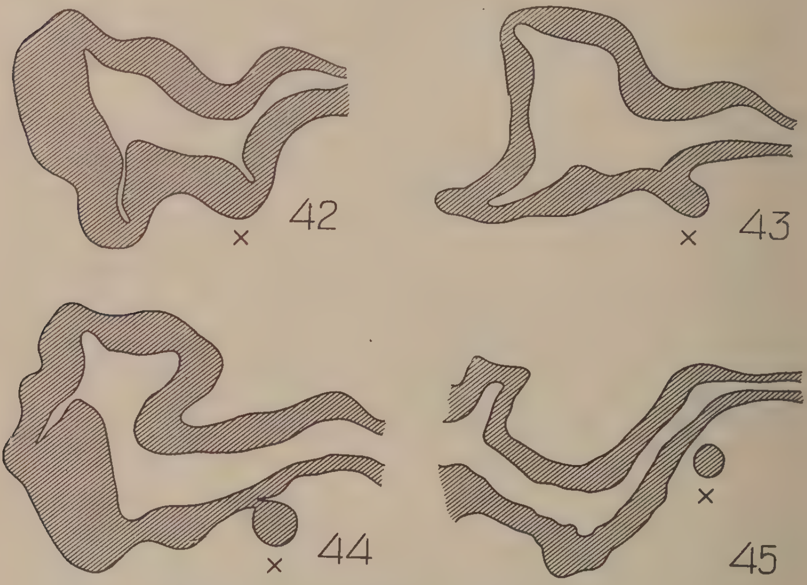
The normal development of the ultimobranchial body has been studied on the material which the Hubrecht Laboratory has collected for the 'Normal table' of *Xenopus laevis* Daudin. The stages 41–56 investigated in this study were prepared by Prof. J. Ariëns Kappers, M.D., Groningen, and stages 57–66 by one of us (Toivonen). The former part of the material was sectioned serially at 10 μ and stained with haematoxylin-eosin. The latter part was serially sectioned at 20 μ and stained with 'Nucplascol' (Grübler-Hollborn). For the more detailed histological study and for the experimental work embryos reared in our laboratory have been used. Their developmental stages are given as Hubrecht Laboratory stage numbers. In the detailed studies Bouin's fluid or formol-alcohol was used as fixative. The thickness of the sections was 6 μ and the staining Heidenhain's azan.

The experiments with I^{131} were performed as follows. The experimental animals were kept for 4 days in a solution containing radioactive iodine (200 μC. I^{131} in 1,000 ml. water). The temperature of the solution was 22° C. Thereafter the thyroid gland and the larynx, together with the surrounding tissues, were taken out and fixed for 3 hours in Carnoy's fluid, the pieces being simultaneously stained with eosin. After embedding in paraffin the blocks were serially sectioned at 10 μ, mounted on slides and covered with a thin layer of celloidin (0·4 per cent. celloidin solution). The preparations were then placed on the film (Guilleminot Collodium 4). Exposure lasted 14 days. After the autoradiographs had been made, the preparations were stained in the usual way.

RESULTS

Formation and development of the ultimobranchial body

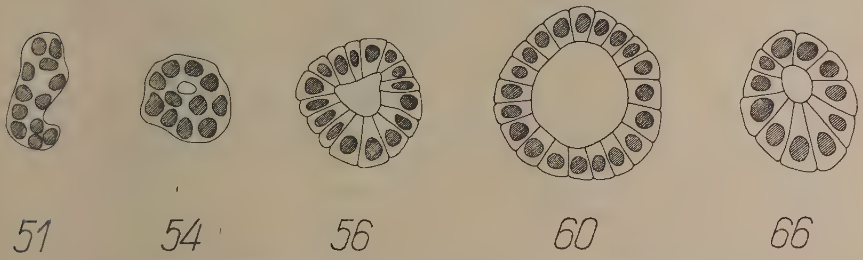
The first developmental stage of the ultimobranchial body is seen in stage 40. On the caudal wall of the pharyngeal cavity, on the median and caudal side of the fifth visceral pouch, a small pit with a pointed bottom is seen. The epithelium has not yet developed a fold at this site. In the following stage the pit has deepened and forms a finger-like lumen which is encircled by the fold of entoderm. The picture corresponds wholly with that of a young visceral pouch, in this case the sixth. It is unique in being directed medioventrally and thus does not reach the ectoderm as do the other visceral pouches. In stage 42 (Text-fig. 1) the sixth visceral pouch is still seen approximately unchanged in size and form. In the following stage (43) the formation of the ultimobranchial body itself begins. The finger-like lumen of the sixth pouch has narrowed to a slit and the entoderm fold becomes thinner at its base and separates from the pharyngeal wall (Text-fig. 1; Plate 1, fig. 1).



TEXT-FIG. 1. The formation of the ultimobranchial body from the sixth visceral pouch. The sixth visceral pouch (stage 42), marked in the figure with a cross, separates itself, forming an entodermal islet (stage 45), the anlage of the ultimobranchial body.

In stage 44 the ultimobranchial body is seen for the first time as an independent organ anlage. The lumen of the sixth visceral pouch is closed and the undifferentiated entodermal cell ball thus formed is connected with the pharyngeal wall only by a thin stalk (Text-fig. 1; Plate 1, fig. 2). In the following stage the connexion is broken and the body is found as a separate cell group laterally to the laryngeal aditus (Text-fig. 1). The islets gradually grow and in stage 47 form solid balls, about 35 μ in diameter. Thereafter the balls are dispersed over a wide space, forming small, solid islets of 8–10 μ in diameter. In stages 48–50 no differentiation of these islets is to be observed. The cells are rather small, the limits of the cells indistinct, and the nuclei large and poor in chromatin.

The differentiation of the ultimobranchial cell islets starts in stage 51 (Text-fig. 2; Plate 1, fig. 3). The cells orientate themselves at the periphery of the ball, forming short strands or segments. The cell limits are still indistinct and one cannot remark any distinct formation of follicles. In stage 52 the cell limits have become clearer and at the same time some nearly complete follicles with a very small lumen can be observed. Both here and in the following stage the majority of the islets are still without a lumen. In stage 54 (Text-fig. 2) the follicles have grown in size and number. The lumen has pronounced limits, but it is still narrow, the diameter always being smaller than the height of the epithelium. In a transverse section the wall shows 8–10 cells. In the lumen one can now and then observe an indistinct content which cannot, however, be considered with certainty to be a secretion. The size and form of the follicles are fairly uniform, but their number in different individuals varies greatly. In stage 56 (Text-fig. 2; Plate 1, fig. 4) the follicles are typical and their lumen has widened, being approximately equal to the height of the epithelium. The wall is composed of columnar epithelium, the large nuclei are generally localized in the proximal part of the cells. The epithelium appears to have a basal membrane. It is not possible to state with certainty that a secretion is present, but in the follicles one may often observe a clear, faintly staining content. In the apex of some epithelial cells there are small balls, which may be apocrine secretion granules. The follicles are surrounded by a rich capillary net which in stages 57–58 is further enlarged.



Text-fig. 2. Follicles of the ultimobranchial body at different stages of development. Formation (stages 51–54), growth (56–60), and reduced form (stage 66). × 500.

After the ultimobranchial body has reached the developmental stage described above, the following stages reveal no noteworthy changes. The size of the follicles and the ratio of the epithelium and lumen are rather variable, but a clear increase in size is observable both in the whole follicle and in the lumen (Text-fig. 2; Plate 1, fig. 5; Plate 2, fig. 7). The follicles still show the above-mentioned content, which is faintly stained with methylene blue, but in the epithelial cells there are no definite signs of any secretion. A great variability in the number of follicles is seen, as earlier. This stage of slow growth extends until the last stages of metamorphosis, stages 63–64. Thereafter a phase in the development of the ultimobranchial body is observed which for several reasons may be considered as a decline. The follicles no longer increase in size, the earlier wide lumen becomes narrower and the number of the follicles is reduced. In stage 66 as well as in the young adult individuals studied only 2–5 follicles are found and in their narrow lumen no secretion is seen (Text-fig. 2; Plate 1, fig. 6; Plate 2, fig. 8).

Features of the development of the thyroid gland

In trying to elucidate the significance of the ultimobranchial body and especially its relation to the thyroid gland and epithelial bodies, it is important to compare its development with that of these endocrine glands, whose function is known.

In stage 40, where the sixth visceral pouch is found for the first time, the anlage of the thyroid gland is seen as a paired cell strand on both sides of the hyoid crest. The connexion with the thyroglossal duct is lost but at the cranial part the duct itself is still present. The cell strands show no cell differentiation. Not until stage 48 do the cells form 4–5 ill-defined lobes. In the following stage the beginning of follicle formation is seen in the same way as in the ultimobranchial body in stage 51. In stage 50 the follicles are already well defined and contain faintly staining material, but only in stage 51 is there any hint of a secretion. At this time the wall of the follicles is complete, the lumen contains strongly staining colloid and there are resorption vacuoles at the site of the epithelial cells. Measurements reveal that the organ continues to increase in size until stages 62–63. At the end of metamorphosis the thyroid gland is continuously regressing in size and in stage 66 its size is only about 70 per cent. of what it was in stage 63.

Experiments with radioactive iodine show that the thyroid gland is already active in stage 51. The storage of I^{131} is proved by the distinct autoradiograph.

It should further be stated that throughout development the situation of the thyroid gland is much more cranial than that of the ultimobranchial body. Even though their distance apart decreases at the end of metamorphosis, no sign of a connexion between them can be observed.

Features of the development of the epithelial bodies

The first phase in the development of the epithelial bodies (parathyroid glands) is observed in stage 43, when a small thickening is seen in the ventral epithelium of the third and fourth visceral pouches. The thickening grows to form club-like anlagen remaining in connexion with the epithelium of the visceral pouches. They begin to differentiate in stages 52–53. Their cells then orientate themselves to give the characteristic concentric formation and a thin membrane composed of squamous epithelial cells is formed around the gland. Except for the gradually increasing size of the glands there are no noteworthy changes until the end of metamorphosis. In stages 59–60 the stalk connecting the epithelial bodies with the epithelium of the pouches is broken and their club-like form is rounded off. Thereafter the glands are nearly spherical. Some of the older tadpoles show 2–3 accessory bodies, which are always located in the direct neighbourhood of the main gland and inside the same capsule.

Experiments with I^{131}

The question of the relation between the ultimobranchial body and the thyroid gland, which has constantly engaged the attention of investigators, has been a source of controversy which has remained unsettled. As Kingsbury (1939) points out, the thyroid-like structure of the gland and the observation of a colloidal secretion in the follicles are not sufficient to prove that the gland has a thyroid-like activity or is actually a separate thyroid gland. He continues: 'The final test would be whether the cell metabolism is of such a character that substances binding iodine are produced.' Such an experiment has been published by Gorbman (1947). He studied the thyroid gland of a young rat, using the autoradiograph technique and stated that here and there the thyroid gland tissue was not able to store iodine. These iodine-negative regions, which also differed morphologically from the thyroid gland proper, are, according to Gorbman's hypothesis, formed by the ultimobranchial tissue. Since, however, the origin of these iodine-negative regions is not known with certainty, and, further, the experiment was performed on one individual only, it needs confirmation. The species we have investigated has an ultimobranchial body which is distinctly separate from the thyroid gland and it is therefore well suited to such a study. Since the histological studies showed that the ultimobranchial body is most active during metamorphosis, tadpoles of different stages were investigated.

The experiments were performed using the method already described. As a control an autoradiograph of the thyroid gland of each animal was also taken. The number of animals investigated was six, including two individuals each from stages 51 and 60 and two young adult animals. The result was without exception negative. While the thyroid gland of each individual gave a distinct autoradiograph, the ultimobranchial body never stored any iodine (Plate 2, figs. 9–12).

DISCUSSION

In the present study an attempt has been made to throw light on two questions concerning the ultimobranchial body: what is the function of this organ and what is its relationship to the endocrine derivates of the pharyngeal cavity, the thyroid gland, and the epithelial bodies?

On the basis of histological studies Terni (1927) considers the ultimobranchial body to be an active gland. Watzka (1933) has performed extensive comparative investigations and considers that the structure of the ultimobranchial body in lower vertebrates clearly indicates the internal secretory activity of the gland. Dudley (1942), on the basis of her experiments on birds, formed the opposite opinion and suggests that the organ has no secretory function. The results presented in this paper, however, support the idea that the ultimobranchial body may have its own specific function. The very development of the organ is in conflict with the concept of a purely rudimentary remnant of a visceral pouch. After becoming separated from the epithelium of the pharyngeal cavity, the 'remnant' continues to grow and differentiates into a separate organ which displays a regular structure and which is observed in all individuals studied. Further, it cannot be contested that the structure of the ultimobranchial body, as described, is clearly glandular. The follicles with a relatively wide lumen, the columnar epithelium, and the secretion granules observed in its cells, as well as the wide capillary net surrounding the follicles, go to prove, in our opinion, a secretory function. The histological investigation shows, however, that activity during development varies. It seems not to begin until the emergence of the hind limbs, attaining its maximum near the end of metamorphosis after the emergence of the fore limbs, and weakening thereafter simultaneously with the regression of the tail. Compared with that of the embryo, the ultimobranchial body of the adult is decidedly atrophied, and the typical signs of activity, secretion granules, wide lumen, and secretion, are lacking. Thus the functional significance of the ultimobranchial body after metamorphosis remains an open question, but it is evidently in any case reduced.

The other proposed question is the relation of the thyroid gland and the epithelial bodies to the ultimobranchial body. The relation was of especial interest to the investigators mentioned in the Introduction who studied the development of the ultimobranchial body in mammals. Special attention must be given here to the theory according to which the ultimobranchial body may form active thyroid tissue (Badertscher, 1918; Rogers, 1929; Weller, 1933; Massart, 1940). On the basis of his experimental results, van Dyke (1944, 1949, 1955) has stated that during development the ultimobranchial body may form thyroid tissue and that after a partial destruction of the thyroid gland it acts as a thyroid growth centre, forming new thyroid tissue. After birth, however, the ultimobranchial body atrophies and it is found in the thyroid gland as atrophied follicle-like cysts. This ultimobranchial tissue in the thyroid gland may be the source of some thyroid tumours (van Dyke, 1955). The present results on the frog, where the ultimobranchial body is formed as a separate organ, however, give no hint of a connexion between this organ and the thyroid gland. The ultimobranchial body, according to some authors a 'lateral thyroid' (Massart, 1940), is in no developmental stage in contact with the thyroid gland proper, neither does it ever store iodine like the thyroid gland. Although its structure closely resembles that of the thyroid gland, there is never in its follicles any substance staining like the thyroid colloid. Further, the ultimobranchial body is clearly formed later than the thyroid gland. These observations may be sufficient to prove that in the species studied the ultimobranchial body does not form thyroid tissue nor does it function like the thyroid gland.

Of course, these results cannot be generalized with certainty, but it is improbable that this organ has an entirely different activity in different vertebrates. It is another question whether the ultimobranchial body of different authors is indeed the same organ. As mentioned already, its formation in different vertebrates is very variable (from the fourth to the sixth visceral pouch), and it is not certain whether the organs described have anything in common apart from a similar structure and the same name.

Dudley (1942), in birds, has stated that during development the ultimobranchial body forms accessory parathyroid glands. Although the species we have studied also has accessory epithelial bodies, they are probably not derived from the ultimobranchial body. The distance between the two organs throughout development is relatively great and the accessory epithelial bodies are in addition located inside the same capsule as the main gland. A more natural explanation in this case is that a part of the epithelial gland rudiment has isolated itself and developed separately.

SUMMARY

The authors have investigated the formation and normal development of the ultimobranchial body and its ability to store iodine in *Xenopus laevis* Daudin. In this species the ultimobranchial body develops from the base of the sixth visceral pouch and forms laterally to the larynx a paired organ in which a limited number of distinct follicles are seen. The organ is formed at the beginning of metamorphosis, attains its maximal size in the middle of it, and partially degenerates at the end of metamorphosis. On the basis of the results presented it is suggested that the ultimobranchial body has its own specific secretory activity, which is evidently most highly developed during metamorphosis and weakens thereafter. Investigations with radioactive iodine (I^{131}) have shown that the organ does not act as a thyroid gland. Neither does the ultimobranchial body form thyroid tissue or accessory epithelial bodies (parathyroid glands) during development.

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EXPLANATION OF PLATES

PLATE 1

FIGS. 1–2. The formation of the ultimobranchial body (marked with an *X*) from the sixth visceral pouch. Stages 43 and 44. Haematoxylin-eosin.

FIGS. 3–4. The ultimobranchial body (*X*) in stages 51 and 56. Haematoxylin-eosin.

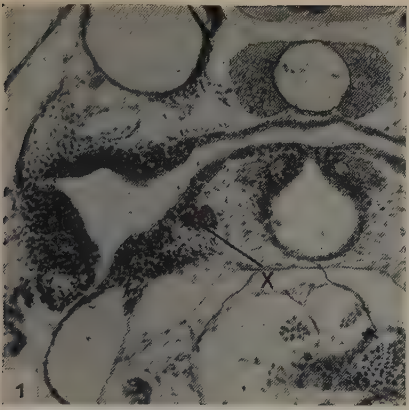
FIGS. 5–6. The ultimobranchial body (*X*) in stages 60 and 66. Heidenhain's azan.

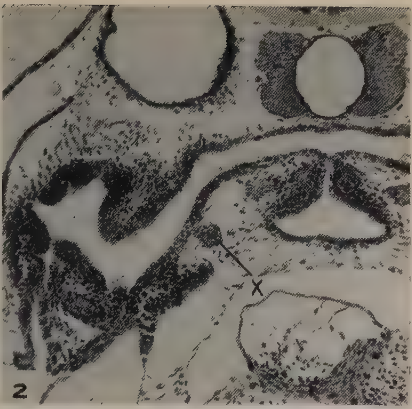
PLATE 2

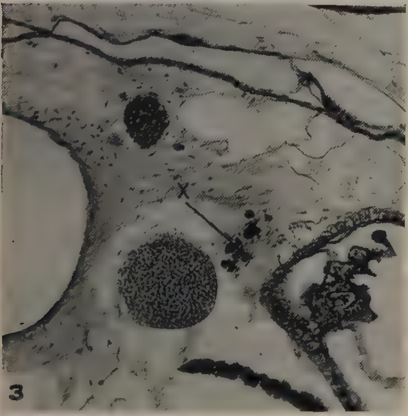
FIGS. 7–8. Follicles of the ultimobranchial body in stages 60 and 66. Heidenhain's azan. × 300.

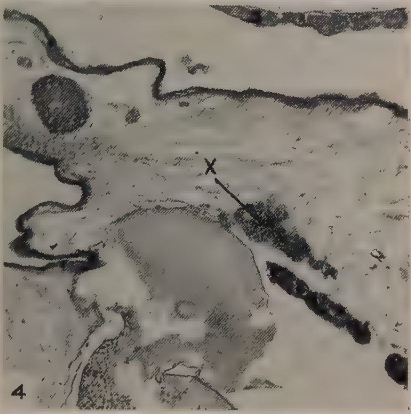
FIG. 9–12. Ultimobranchial bodies and thyroid glands of the same animal (stage 60) (Figs. 9, 11) and the corresponding autoradiography films (Figs. 10, 12).

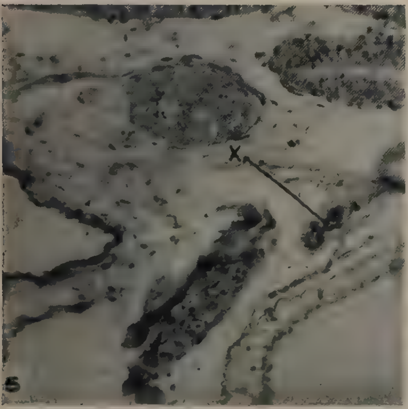
(Manuscript received 16:iii:55)

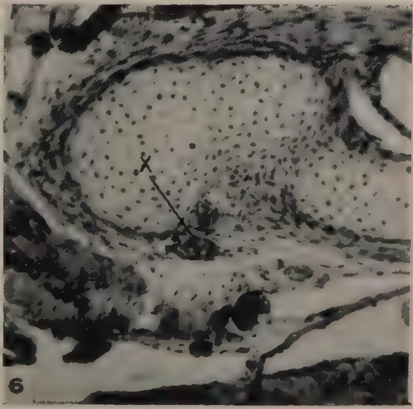












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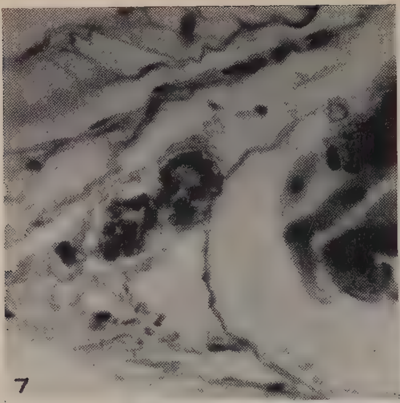
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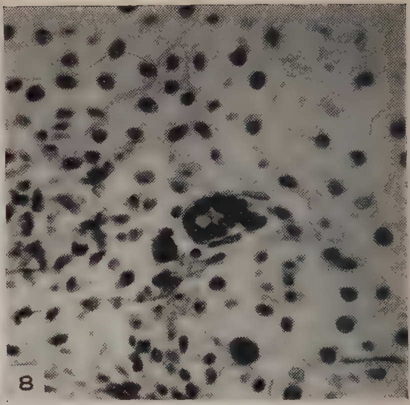
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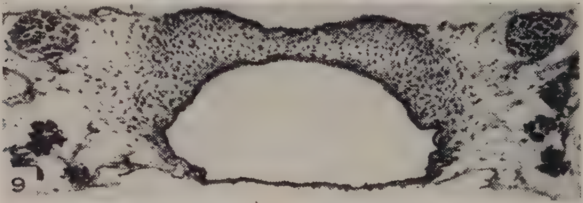
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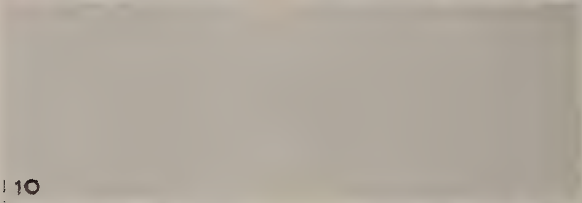
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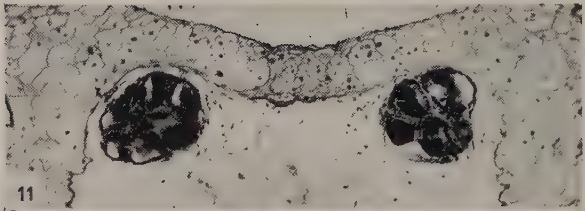
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